Regulation of human Leydig cells : comparison with the rat.

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Contents

Abbreviations

3β-HSD	3β-hydroxy steroid dehydrogenase
ABP	androgen binding protein
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
DBI	diazepam binding inhibitor
DHT	dihydrotestosterone
EDS	ethane dimethane sulphate
ES	elongate spermatids
FSH	follicle stimulating hormone
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
HCZ	hexaconazole
IF	interstital fluid
KCZ	ketoconazole
LH	luteinising hormone
LHRH-A	luteinising hormone releasing hormone agonist
M199E	medium 199 containing Earle's salts
M199H	medium 199 containing Hank's salts
MAA	methoxy acetic acid
PDGF	platelet derived growth factor
POMC	pro-opiomelanocortin
PS	pachytene spermatocytes
PSG	pregnancy specific glycoprotein
RS	round spermatids
SPARC	secreted proteinand rich in cysteine
STF	seminiferous tubule fluid
STM	seminiferous tubule conditioned medium
TKD	tralkoxydim

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Declaration

The experiments described in this thesis were the unaided work of the author except where acknowledgement is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

The major aim of this thesis was to study the regulation of testosterone production in the human testis. The technique used to isolate human Leydig cells was improved, so that Leydig cells responsive to hCG were obtained consistently in large yields. There was marked subject variation in both the basal levels of testosterone produced and in the responsiveness of the cells to hCG. Sertoli cells are believed to modulate Leydig cell function via the action of secreted factors. The nature of these factors is not known, although in the rat GnRH and AVP are potential candidates. Basal testosterone production by isolated rat Leydig cells is stimulated by GnRH and AVP, and comparable effects on isolated human Leydig cells were demonstrated. This evidence of comparable control mechanisms in the rat and human testis was supported by the finding that proteins secreted by isolated rat and human Leydig cells were remarkably similar when analysed by 2-dimensional SDS-PAGE. In addition, rat and human Leydig cells showed comparable responses to the deleterious effects of a variety of toxicants *in vitro*.

A morphological examination of the relationship between Leydig cells and other components of the human testis distinguished light and dark Leydig cells on the basis of differential toluidine blue staining. Morphometric analysis of testicular cellular components was correlated with the ability of isolated Leydig cells to produce testosterone *in vitro*. No relationship was found between either the total number of Leydig cells, or the proportion of light and dark Leydig cells per testis and testosterone production or hCG responsiveness *in vitro*. The number of light or dark Leydig cells showed significant positive correlations with different testicular components, perhaps indicating specific roles for these two subpopulations.

The cascade of regulatory interactions between germ cells, Sertoli cells and Leydig cells was investigated in the rat. The absence of pachytene spermatocytes or round spermatids *in vivo* was associated with increased testosterone production *in vitro* by Leydig cells subsequently isolated from the affected testes and was also associated with the secretion of a novel Leydig cell protein *in vitro*.

Interactions between rat Leydig cells and stage-dissected seminiferous tubules were studied using *in vitro* co-cultures. The role of germ cells was determined by isolating seminiferous tubules from control rats or rats in which a single germ cell type had been depleted selectively. Seminiferous tubules at stages II-V inhibited basal testosterone production by isolated Leydig cells. Metabolism of testosterone by seminiferous tubules was highest at stages VI-VIII, stage VII being the androgen-dependent stage.

In conclusion, these studies have demonstrated the isolation of human Leydig cells, which, *in vitro*, respond to the same stimuli as rat Leydig cells, and secrete many very similar proteins, suggesting the modulation of Leydig cell function in the two species is comparable. In the rat, one mechanism of controlling Leydig cell function may involve modulation by specific germ cell types, presumably mediated via Sertoli cells. The morphological analysis of the human testis, and the correlations subsequently made support the existence of such an interaction in man.

1. Introduction.

In the West 5% or more of the male population are subfertile. A recent review of studies published between 1938-1991 revealed a marked decline in sperm counts in men without a history of infertility. (Carlsen *et al.*, 1992). One factor believed to have contributed to this decline is increased exposure to various environmental chemicals (Sharpe, 1992b). Both studies emphasised the present lack of understanding of the mechanisms controlling spermatogenesis, which in turn limits the treatment of male infertility. Thus it is essential that the processes that control testicular function are more thoroughly investigated. Testosterone is an essential requirement of spermatogenesis, therefore the mechanisms that regulate testosterone production are potential sites for malfunctions that could result in impaired sperm output.

Increasingly, evidence suggests that testicular function is controlled by many complex cell-cell interactions (for review see Skinner, 1991, Sharpe, 1992). The complexity of the interactions that occur in the rat testis was demonstrated by Onoda *et al.* (1991). The addition of conditioned medium from rat Sertoli cell cultures to cultures of rat Leydig cells stimulated testosterone production. If the Sertoli cells had been co-cultured with pachytene spermatocytes, the ability of the Sertoli cell conditioned medium to stimulate Leydig cell steroidogenesis declined. The study thus demonstrated that specific germ cell types, via an action on Sertoli cells, can influence Leydig cell function.

In fact, there is evidence in the rat that Leydig cells respond to many alterations in cellular composition and cell-cell interactions. Experimentally induced damage to the seminiferous epithelium results in Leydig cell hypertrophy (Aoki & Fawcett, 1978, Risbridger *et al.*, 1981, Jegou *et al.*, 1984), and the production of testosterone by isolated Leydig cells is influenced by co-culture with Sertoli cells or by the addition of Sertoli cell conditioned medium (Verhoeven & Cailleau, 1986, 1990). Disruption of such intercellular regulatory pathways may be one possible cause of infertility. However, very little is known about the control of testicular function in man, and it is not clear whether the rat and human testis would have similar local regulatory mechanisms.

The primary purpose of this thesis was therefore to investigate the regulation of testosterone production in the human testis. The secondary aim was to study the cascade of regulatory interactions between germ cells, Sertoli cells and Leydig cells that may occur in the rat testis. Therefore the first experimental chapter describes the isolation and culture of human Leydig cells, and characterises their performance *in vitro*. Subsequent chapters assess both the effects of putative paracrine regulators of rat Leydig cell function on testosterone production by human Leydig cells and the comparative susceptibility of rat and human Leydig cells to toxicant effects. Proteins secreted by rat and human Leydig cells under basal and hCG-stimulating conditions were also examined.

The nature of cell-cell communication in the rat testis was investigated using an *ex vivo* technique. Specific germ cell types were depleted from the testis *in vivo*. Leydig cells were subsequently isolated from these testes and the effect of this depletion on their ability to produce testosterone *in vitro* was examined. Further examination of cell-cell interactions involved co-culturing Leydig cells with seminiferous tubules known to be at particular stages of the spermatogenic cycle. The influence of germ cells on the interactions between the epithelium and the interstitium was assessed by isolating seminiferous tubules from both control and germ cell-depleted rats.

2. Review of the literature

2.1. Introduction

The mammalian testis has two functions, the production of male gametes, and the production of male sex hormones. These two functions are very closely related; spermatogenesis can only proceed in the presence of adequate levels of androgens and the successful delivery of sperm depends on the development of secondary sexual characteristics and normal sexual behaviour, which are both controlled by androgens.

The first person to describe the endocrine role of the testis was Berthold in 1849 (see Burger & de Kretser, 1981 for translation) who recorded that castration of cockerels resulted in decreased comb size and less aggressive behaviour. This change did not occur if the testes were transplanted to the abdominal cavity. Berthold concluded that the testis was able to affect the blood so that it in turn could affect the entire organism. In fact, testicular steroids with the ability to restore sexual characteristics in castrated animals ('androgens') were not isolated until the 1930s and 40s (see Setchell, 1978 for review).

Although Leydig cells were first described in 1850, their function remained unknown until 1903 when Bouin & Ancel suggested that Leydig cells were the site of production of the male sex hormones. This was based on the observation that despite the induction of tubular atrophy by experimental cryptorchidism, male characteristics remained unaffected, suggesting that the undamaged interstitial tissue was the source of androgen. The function of Leydig cells was not established conclusively until Hall *et al.* (1969) separated tubular and interstitial tissue and demonstrated that *in vitro*, interstitial tissue could convert ^{3}H-cholesterol to testosterone but tubular tissue could not. Also, when ^{3}H-cholesterol was injected i.v. into male rats and interstitial and tubular tissues were separated after death, the radioactivity in interstitial tissue significantly exceeded that measured in tubular tissue (Parvinen *et al.*, 1970). Cooke *et al.* (1972) measured both the endogenous testosterone concentrations in isolated interstitial and tubular tissue and the testosterone concentrations during *in vitro* incubations. The highest

concentration of testosterone was in interstitial tissue and this concentration increased during the incubation, suggesting *de novo* synthesis of testosterone from endogenous precursors. This increase in testosterone concentration *in vitro* was not observed with seminiferous tubular tissue. Previously the key steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) had been localised to Leydig cells (Levy *et al.*, 1959) and Woods & Domm (1966) had used fluorescent labelled antibodies to localise testosterone to Leydig cells. These combined approaches established the Leydig cell of the rat as the producer of sex steroids. Kawano *et al.* (1973) showed that the interstitial tissue of the adult human testis was the major site of conversion of pregnenolone to testosterone.

Sertoli cells were first described in 1865, and while their close association with the spermatogenic process has always been appreciated, specific functions of Sertoli cells have only been described in the past 20 years. These will be discussed in section 2.3.2.4. and 2.3.2.5.

The role of the pituitary in controlling testicular function was demonstrated by Smith (1930), who showed that hypophysectomy caused testicular regression and abolition of spermatogenesis. Fevold *et al.* (1931) demonstrated that the anterior lobe of the pituitary gland secreted two substances which could regulate ovarian development. Based on their effects on the ovary these substances were named follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In 1936 Greep *et al.* investigated the effects of LH and FSH on the gonads and secondary sexual structures of normal and hypophysectomised immature male rats. In 28 day old hypophysectomised rats in which the gonads and secondary sexual structures showed marked atrophy, FSH had a strikingly selective effect on tubular histology, with the seminiferous epithelium increasing in size. FSH had no effect on the size of the seminal vesicles or prostate. In contrast, LH caused hypertrophy of the interstitial tissue and was also able to increase the size of the accessory organs. Similar distinct actions of LH and FSH were later demonstrated in adult rats (Greep & Fevold, 1937) and in the mouse (Randolph *et al.*, 1959). The ability of human pituitary gonadotrophins to restore fertility to hypophysectomised adult men was also demonstrated (Gemzell & Kjessler, 1964, MacLeod *et al.*, 1966). In 1967 Mancini *et al.* found that when injected into rats, labelled LH

concentrated in Leydig cells, while labelled FSH accumulated primarily in Sertoli cells.

Hypothalamic extracts from several species, including man, were shown to contain a substance(s) capable of stimulating the release of LH and FSH from the pituitary (McCann & Ramirez, 1964, Schally *et al.*, 1970). It was initially thought that two separate substances, 'luteinizing hormone releasing hormone' (LHRH) and 'follicle-stimulating hormone releasing hormone' (FSHRH) were responsible for such effects, but preparations of LHRH of increasing purity were always able to stimulate the release of both LH and FSH (Kastin *et al.*, 1969, Schally *et al.*, 1971). While this suggested that only one releasing hormone existed, this was only confirmed with the determination of the structure of a hypothalamic peptide with both LHRH/FSHRH activities (gonadotrophin releasing hormone, GnRH; Matsuo *et al.*, 1971, Schally *et al.*, 1971). Synthetic GnRH was shown to increase plasma LH and FSH in castrated rats (Matsuo *et al.*, 1971) and in normal men (Yen *et al.*, 1972).

In rats removal of the testes was found to result in marked increases in plasma LH and FSH levels. However, subsequent administration of testosterone and oestradiol returned gonadotrophin levels to normal (Swerdloff & Walsh, 1973). This negative feedback control allows the testis to maintain tonic serum concentrations of gonadotrophins.

In summary, the research discussed above describes how endocrine secretions from the hypothalamus, anterior pituitary and testes control the male reproductive system. GnRH controls the secretion of the gonadotrophins, LH and FSH by the anterior pituitary. Through actions on the Sertoli cells, FSH is involved in testicular development at puberty and in the adult may have a co-operative role with testosterone in driving spermatogenesis. LH stimulates Leydig cells to secrete testosterone and other androgens. Testosterone is responsible for the development and maintenance accessory sex organs and for the maintenance of spermatogenesis. Further, testosterone is able to regulate the level of its own production through a negative feedback effect on the anterior pituitary. Together the studies discussed above define the hypothalamic-pituitary-testicular axis. The regulation and coordination of this axis is discussed in more detail in the following section.

2.2. Endocrine control of the testis

2.2.1. LH

LH belongs to a family of glycoprotein hormones the common structure of which consists of 2 non-covalently linked subunits, α and β (Liao *et al.*, 1969). Other members of this family are thyroid stimulating hormone (TSH), FSH and hCG. The 92 amino acid α subunit is common to all these hormones (Liao & Pierce, 1971), but each has a unique β subunit which confers specific biological activity via target tissue receptor recognition (Pierce *et al.*, 1971). Until recently the individual subunits in the dissociated state were thought to have no intrinsic biological activity (Catt *et al.*, 1973). However, by using synthetic preparations of α subunit, Erickson *et al.* (1990) demonstrated that peptides corresponding to regions of hCG binding were able to stimulate steroidogenesis in crude preparations of rat Leydig cells. The authors propose that these regions of the α subunit align with the β subunit and are involved in binding the LH receptor, as well as the ligand. LH binding to its receptor may occur in two stages, whereby initial binding of the β subunit ensures specificity, while subsequent binding of the α subunit confers stability (Metsikko *et al.*, 1990).

LH is secreted in episodic bursts by gonadotrophs in the pars distalis of the anterior pituitary with each species having its own frequency of LH pulses. In man LH pulses occur at approximately 90 minute intervals (Nankin & Troen, 1971, Santen & Bardin, 1973). In the rat the timing of LH pulses appears to be very variable (Gay & Sheth, 1972, Ellis & Desjardins, 1982).

2.2.2. Hypothalamic control of LH secretion

The episodic nature of LH secretion is controlled by the pulsatile secretion of GnRH from the arcuate nucleus of the hypothalamus (for review see Knobil, 1980). The pattern of GnRH secretion was described by Carmel *et al.* (1976) who measured pulsatile GnRH release into the portal blood of female Rhesus monkeys and noted peak intervals of between 1-3 hours. The fluctuations in GnRH levels appeared to be associated with increases

in plasma LH levels. Ellis *et al.* (1983) administered either ovine anti-GnRH antiserum or a GnRH antagonist to castrated male rats and showed that both treatments were able to abolish LH pulses. In man, GnRH extracted from plasma was found to release LH from incubated rat hemipituitaries (Elkind-Hirsch *et al.*, 1982). Infusions of synthetic GnRH into the antecubital vein of both men and women has been shown to result in increased serum LH and FSH levels (Yen *et al.*, 1972, Dufau *et al.*, 1976, Elkind-Hirsch *et al.*, 1982).

GnRH has been shown to bind to specific high affinity receptors on gonadotroph cell membranes (Theoleyre *et al.*, 1976). GnRH receptors do not appear to use the adenylate cyclase/cAMP second messenger pathway (Clayton *et al.*, 1978, Benoist *et al.*, 1981). In fact, it has since been shown that following receptor binding, GnRH stimulates phosphoinositide turnover for which no rise in intracellular calcium levels is needed. The activation of phospholipase C (PLC) is believed to be mediated via a pertussis toxin-insensitive G-protein. The formation of inositol-1,4,5-trisphosphate (IP_3) enhances calcium release from intracellular stores and this in turn may be responsible for the translocation of subspecies of protein kinase C (PKC) from the cytosol to the membrane. In turn activated PKC phosphorylates substrate proteins which activate secretory reactions and participate in gonadotrophin gene expression. An influx of extracellular calcium through membrane ion channels further elevates the intracellular calcium concentration, contributing to the sustained phase of gonadotrophin release. GnRH also triggers the release of arachidonic acid (AA) and the formation of lipoxygenase and/or epoxygenase products which are involved in exocytosis (for review see Naor, 1990).

2.2.3. Site of LH action

In 1936 Greep *et al* demonstrated that LH caused proliferation of interstitial but not tubular tissue in hypophysectomised immature male rats. Later, LH labelled with various histochemical agents was localised to Leydig cells in the testis of adult rats (Mancini *et al.*, 1967). Radiolabelled LH has also been shown to accumulate in rat interstitial cells (de Kretser *et al.*, 1969) due to the presence of specific high affinity, low capacity

receptors (Catt *et al.*, 1971). Specific, high affinity, low capacity binding of ^{125}I-hCG to adult human testicular homogenates has also been demonstrated (Hsu *et al.*, 1978, Sharpe *et al.*, 1980).

The LH receptor is localised at the cell surface and is a member of a family of receptors which all contain 7 transmembrane spanning domains. The receptor has an large extracellular domain which comprises the hormone binding site. (see Bahl, 1977, Metsikko *et al.*, 1990 for review). A 'hand-and-glove' model for hCG-receptor binding has been recently proposed (Schwarz *et al.*, 1991).

The human LH receptor was isolated and cloned from human ovarian RNA by Minegish *et al.* (1990). The extracellular domain showed 85% homology to the rat LH receptor (McFarland *et al.*, 1989) and the putative membrane spanning domain had 90% homology with the LH receptor found in the rat. The importance of the heterologous regions was demonstrated by Jia *et al.* (1991). The authors found that when human ovarian cDNA for LH was transfected into a human embryonic kidney cell line, the expressed receptors would recognise human LH/CG but not rat, equine or ovine LH/CG. In contrast the rat LH receptor is able to recognise LH/CG from all of the above species (Farmer *et al.*, 1977).

2.2.4. Mechanism of LH action

In 1977, Dufau *et al* demonstrated that the incremental testosterone response of purified rat Leydig cells to increasing concentrations of hCG, was accompanied by a simultaneous increase in the levels of endogenous cAMP bound to the regulatory unit of protein kinase. The production of cAMP is dependent on the activation of membrane bound adenylate cyclase (Kuehl *et al.*, 1970, Dorrington & Fritz, 1974, Mendelson *et al.*, 1975). The binding of cAMP to the regulatory unit of protein kinase causes the enzyme to dissociate, and the freed catalytic subunit is able to activate specific proteins by phosphorylation (Podesta *et al.*, 1978, Catt & Dufau, 1976). Similarly, in the human, hCG injected directly into the testis was found to increase the spermatic vein concentrations of both cAMP and testosterone. The rise in cAMP levels preceded the rise in testosterone levels (Yasukawa *et al.*, 1981).

In this way LH increases the activities of steroidogenic enzymes such as cholesterol P_{450} side-chain cleavage (Anderson *et al.*, 1985). This enzyme catalyses the conversion of cholesterol to pregnenolone, which is the rate limiting step of testicular steroidogenesis (Waterman & Simpson, 1985). Cholesterol is the precursor for testosterone synthesis and is synthesized in rat Leydig cell mitochondria (Pignataro *et al.*, 1983) from acetate (Morris & Chaikoff, 1959). LH accelerates the entry of cholesterol into mitochondria (Hall *et al.*, 1967). Thus the main effect of LH is to increase the availability of steroid substrate by accelerating the metabolism of cholesterol.

However, it appears that not all the effects of LH are mediated through the cAMP second messenger system. Testosterone production can be stimulated by amounts of LH that have no detectable effects on intracellular cAMP levels (Cooke *et al.*, 1976). Recent studies have shown that low, non-stimulatory levels of LH and cAMP have a marked synergistic effect on testosterone synthesis (Choi & Cooke, 1992), suggesting independent pathways are involved. Prolonged exposure to LH causes Leydig cell desensitization and the cells become refractory to LH stimulation (see below). This desensitization cannot be mimicked by cAMP analogues, but is mimicked by PKC activators such as phorbol esters (Rose & Band, 1988). Further studies led the authors to propose that activation of adenylate cyclase may be dependent on the action of PKC.

In addition, LH has been shown to stimulate the release of arachidonic acid from Leydig cells (Chaudry *et al.*, 1989). The release of arachidonic acid from phospholipids is dependent on the action of either phospholipase A_2 (PLA_2) or PLC. In purified rat Leydig cells, inhibitors of PLA_2 inhibited LH-stimulated steroidogenesis without affecting LH-induced cAMP formation, and PLA_2 was able to stimulate basal and LH-stimulated testosterone production without affecting cAMP levels (Abayasekara *et al.*, 1990). Based on these results a model of LH action has been proposed whereby LH increases the intracellular calcium concentration, either by the opening of membrane ion channels or by release from intracellular stores. The increased calcium levels activate PLA_2, stimulating the formation of arachidonic acid metabolites which can act as second messengers. When the levels of LH are high, cAMP is also stimulated and may play a co-operative role (Cooke, 1990).

2.2.5. Modulation of Leydig cell response to LH

LH is able to down-regulate the number of its own receptors and thus limit its own potential maximal stimulatory effect. Hsueh *et al.* (1976) demonstrated that administration of a single dose of hCG to either immature whole or hypophysectomised rats was followed by a marked reduction in LH binding capacity to testicular homogenates *in vitro*, between 3 and 24 hours later. A similar desensitizing effect of LH on its receptors in the human was implied by the results of an experiment by Saez & Forest (1979). An initial injection of hCG was shown to stimulate plasma testosterone levels in 13 normal male volunteers. Subsequent injections of hCG 24 hours later failed to induce a significant increase in testosterone levels. Iodinated hCG was shown to bind to human testicular tissue obtained from infertile men undergoing biopsy (Sharpe *et al.*, 1980). Tissue obtained from men who had been treated 24 hours earlier with hCG showed significantly lower hCG binding than in comparable untreated men. Thus it appears that hCG is able to negatively regulate Leydig cell LH receptors in man.

The binding of either ovine LH or hCG to rat gonadal homogenates, testicular membrane preparations or purified Leydig cells is followed by a prolonged loss of LH-receptors. This decrease in receptor number is both time and dose-dependent and results in an increased ED_{50} for steroid production. A decrease in the maximal steroid response to subsequent hormonal stimulation is also seen. The time-scale of receptor loss indicates that the decrease in binding cannot be due to receptor occupancy, but is caused by depletion of LH-receptors from the plasma membrane. The disappearance of functional LH receptors is accompanied by a decrease in the ability of LH to stimulate adenylate cyclase activity and cAMP formation in the testis (for review see Catt *et al.*, 1979).

In rat ovarian membrane preparations, down-regulation of the LH receptor was found to involve internalisation and proteolysis by endogenous proteinases (Kellokumpu & Rajaniemi, 1985). In the same system, the protease inhibitor, aprotinin, has been shown to inhibit the association of hCG to its receptor, although it did not affect hCG binding to solubilised receptors (Wilks & Hui, 1987). In the rat the LH receptor exists in a dynamic state of continuous recycling, in which activation of

the transducing system is followed by uncoupling, internalisation and recycling. (Habberfield *et al.*, 1987, Cooke & West, 1992). Isolated rat Leydig cells in culture exhibit continuous proteolytic cleavage of the LH receptor with release of the extracellular domain. In the rat desensitization to LH/hCG does not involve receptor down-regulation (i.e. internalisation and degradation) (West & Cooke, 1991).

2.2.6. Feedback control of LH

Androgens and oestrogens are able to modulate LH secretion through negative feedback effects. Sherins & Loriaux (1973) studied the effects of testosterone and oestradiol on plasma FSH and LH concentrations. Four day continuous infusions of either steroid resulted in plasma FSH, LH and testosterone levels falling below control levels by the end of the infusions. The negative feedback effects of infusions of testosterone and oestradiol on plasma LH levels were confirmed in a later study which had the advantage of using more physiological doses of the two steroids (Stewart-Bentley *et al.*, 1974). As oestrogen can be formed by the aromatisation of circulating testosterone (Longscope *et al.*, 1969), it was not clear which of these steroids was involved in controlling peripheral gonadotrophin levels. Stewart-Bentley *et al.* (1974) demonstrated that LH levels could be suppressed by the infusion of a non-aromatisable androgen such as dihydrotestosterone (DHT). Using the aromatase inhibitor, Δ^1-testolactone to prevent oestrogen formation from testosterone, Marynick *et al.* (1979) confirmed that testosterone was able to suppress serum LH levels independently of its peripheral aromatisation to serum oestrogen. Testosterone has also been shown to have a negative feedback effect on LH secretion in the rat (Ellis & Desjardins, 1982). In fact, in man androgens and oestrogens have been shown to have differential effects on gonadotrophin regulation. Testosterone and non-aromatisable androgens were found to decrease the frequency of LH pulses without affecting the amplitude of the pulses. On the other hand, oestradiol affected LH secretion by decreasing pulse amplitude without affecting their rate of production (Santen, 1975).

2.2.7. FSH

FSH belongs to the same glycoprotein family as LH and is also secreted by pituitary gonadotrophs. FSH and LH share a common α subunit, but the β subunit of FSH is unique (see Gharib *et al.*, 1990 for review). FSH has a higher M_r than LH, due to its having a greater carbohydrate composition. For the same reason the plasma half-life of FSH is longer at 3-4 hours than that of LH, at 1.5-2.5 hours (Coble *et al.*, 1969, Santen & Bardin, 1973). The pattern of FSH secretion, like that of LH, is episodic. However, FSH levels show very small fluctuations in contrast to the frequent secretory spikes of LH (Santen & Bardin, 1973).

2.2.8. Hypothalamic control of FSH secretion

In rats, the release of both LH and FSH from pituitary gonadotrophs is controlled by a single releasing factor, GnRH (Schally *et al.*, 1971). GnRH has been shown to stimulate the release of LH and FSH from enriched rat gonadotroph populations *in vitro* (Benoist *et al.*, 1981). Yen *et al.* (1972) demonstrated that administration of synthetic GnRH to normal adult men elicited a prompt increase in LH levels that was accompanied by a smaller but concomitant rise in circulating FSH levels. Similarly, Santen & Bardin (1973) described small pulsatile changes in serum FSH levels subsequent to GnRH challenge; although small, these increments were found to coincide with LH pulses more frequently than could be ascribed to chance. However, a separate study with normal male subjects, showed that while synthetic GnRH was able to stimulate release of both LH and FSH, the fluctuations in their serum levels were asynchronous (Mortimer *et al.*, 1974). The authors suggest that the asynchronous release of LH and FSH is due to differential effects of GnRH on the mechanisms responsible for the release of LH and FSH from gonadotrophs.

2.2.9. Site of FSH action

The testicular effects of FSH were described by Greep *et al.* (1936). In immature, hypophysectomised rats the effects of FSH were found to be concentrated solely on the seminiferous epithelium. FSH labelled with

ferritin or fluorescent markers was found to localise around the nuclei and in the apical area of Sertoli cells. It was also found in between germinal spaces which were presumed to be cytoplasmic branches of Sertoli cells (Mancini *et al.*, 1967). Means & Vaitukatis (1972) reported binding of ^{3}H-FSH to the seminiferous epithelium, although the specific cell type binding the FSH could not be distinguished.

FSH has no effect on adenylate cyclase activity in interstitial cell preparations, but increases adenylate cyclase activity in seminiferous tubule preparations (Kuehl *et al.*, 1970). FSH was also shown to increase cAMP accumulation in normal, fully regressed hypophysectomised, and cryptorchid rats. As the two latter types have testes that are enriched in Sertoli cell and spermatogonia, either of these cell types was potentially the site of FSH action. However, testicular irradiation can be used to produce rats with remarkably few spermatogonia. These rats show the same response to FSH as seen in the treatment groups described above. Thus it was deduced that the Sertoli cell was the target cell for FSH (Dorrington & Fritz, 1974).

The presence of FSH receptors in the human testis has been demonstrated using testicular biopsy tissue from infertile patients (Namiki *et al.*, 1984). The number of high affinity FSH binding sites was found to decrease in parallel with the degree of spermatogenic impairment seen in the biopsy tissue.

2.2.10. Mechanism of FSH action

FSH binds specifically to Sertoli cells and via increased cAMP production (Kuehl *et al.*, 1970) activates protein kinases and stimulates end responses such as the production of androgen-binding protein (ABP), plasminogen activator and oestrogen (Hansson *et al.*, 1974, Dorrington *et al.*, 1978).
During puberty, FSH is of critical importance in initiating spermatogenesis. The number of FSH binding sites per testis increases during the neonatal period when Sertoli cells are actively dividing. Maximum numbers are attained at approximately 15 days (Fakunding *et al.*, 1976). Passive immunisation of immature rats with an FSH antiserum was found to decrease testicular size and to disrupt the morphology of the seminiferous epithelium (Raj & Dym, 1976).

The role of FSH in the adult rat is less clear cut. Dym *et al.* (1979) reported that administration of FSH antiserum to adult rats did not affect either ABP levels, the morphology of the seminiferous epithelium, or fertility, suggesting that in the adult rat, FSH may not be required for the maintenance of spermatogenesis. In addition, it had previously been shown that while FSH could stimulate cAMP production in testicular slices or seminiferous tubules isolated from immature rats, it had no effect on cAMP levels in tissue preparations from mature rats (Kuehl *et al.*, 1970). In contrast, in adult Rhesus monkeys, active or passive immunisation against FSH impaired spermatogenic function and decreased sperm counts within 4 weeks of immunisation (Wickings & Nieschlag, 1980), suggesting that FSH does have a role to play in spermatogenesis. Using a highly purified, LH-free FSH preparation, Bartlett *et al.* (1989) demonstrated that treating hypophysectomised adult rats with this preparation of FSH maintained testicular weights and numbers of preleptotene spermatocytes, pachytene spermatocytes and round spermatids, at higher levels than seen in untreated hypophysectomised rats. Testosterone implants were also able to maintain spermatogenesis, and combinations of FSH and testosterone maintained spermatogenesis and testicular weights at almost normal levels. The results of these experiments suggest that FSH does have a role in the maintenance of spermatogenesis in adult rats as well as in adult primates. In man induction of FSH deficiency by chronic administration of hCG resulted in partial suppression of sperm production, and replacement of FSH by injection returned sperm production almost to control levels. This suggests that in man, as in the rat and Rhesus monkey, while normal levels of FSH are not an absolute requirement for sperm production, FSH is necessary for the maintenance of qualitatively normal spermatogenesis (Matsumoto *et al.*, 1986).

At all stages of development the actions of FSH and testosterone seem to be highly integrated. A model for the synergistic action of FSH and testosterone in maintaining spermatogenesis was proposed by Sharpe (1989a): FSH determines the number of viable cells to enter meiosis while testosterone controls their subsequent development. FSH alone would therefore be unable to maintain spermatogenesis as the continued development of germ cells would fail due to the absence of androgenic

support. Testosterone, however, as seen in so many studies, would be able to maintain qualitatively normal spermatogenesis, as the supply of differentiated spermatogonia would be reduced but not abolished in the absence of FSH.

FSH is implicated in the control of Leydig cell maturation. For example, in the sexually immature rat testis, the absence of exposure to FSH results in insensitivity to LH, implying that exposure to FSH induces responsiveness to LH (Odell *et al.*, 1973). In addition, Kerr & Sharpe (1985) reported that administration of FSH to immature, hypophysectomised rats resulted in a significantly greater number of Leydig cells compared to the numbers seen in untreated rats. These changes were a consequence of increased hypertrophy of immature Leydig cells to adult type Leydig cells, and of Leydig cell hyperplasia. A cascade is thus initiated whereby FSH is responsible for the differentiation of the adult population of Leydig cells, which become responsive to LH and produce androgens which act locally to support spermatogenesis. These effects of FSH are presumed to be mediated via Sertoli cells as Leydig cells do not have FSH receptors (Means & Vaitukatis, 1972).

The stimulation of cAMP in the rat seminiferous epithelium by FSH varies in a cyclic manner, with the highest degree of stimulation occurring at stages II-VI, and the lowest at stages VII-VIII (Gordeladze *et al.*, 1982, Nikula *et al.*, 1990). These changes coincide with cyclic changes in the activity of PKC, which has been shown to modulate cAMP production in cultured Sertoli cells (Nikula *et al.*, 1987). The authors suggest that the variation in FSH/PKC response with the stage of the spermatogenic cycle is possibly due to the paracrine modulation of Sertoli cell function by germ cells.

2.2.11. Feedback control of FSH

The increases in LH and FSH levels caused by castration can be prevented by administration of testosterone or oestradiol. In the rat, there is some evidence that testosterone preferentially suppresses LH, while oestradiol has parallel effects on LH and FSH (Swerdloff & Walsh, 1973). Sherins & Loriaux (1973) described how administration of either testosterone or oestradiol to normal adult men suppressed both plasma FSH and LH

concentrations. Suppression of FSH levels by testosterone independent of its aromatisation to oestrogen has been demonstrated (Marynick *et al.*, 1979). However, in cases of disrupted spermatogenesis FSH is selectively elevated. Morphological investigation of testicular biopsy tissue from patients with oligospermia or azoospermia, revealed that when spermatogenesis was arrested prior to spermatid formation, serum FSH concentrations were above normal. However, when spermatids were observed in the biopsy tissue, FSH levels were within the normal range (Franchimont *et al.*, 1972). The authors suggested that the transformation of spermatocytes to spermatids induces the formation of a substance which depresses FSH secretion. Recent support for this interpretation comes from the study by Allenby *et al.* (1991) which showed that secretion of inhibin was reduced only by depletion of elongate spermatids from the seminiferous epithelium and this led to an increase in blood FSH levels. Similar associations between elevated serum FSH levels and testicular damage were also described by de Kretser *et al.* (1974), who found that the most significant correlations were between serum FSH and the mean number of spermatogonia per testis. Krueger *et al.* (1974) used chronic vitamin A deficiency to cause germ cell degeneration. No increase in FSH levels was seen when only spermatogonia and Sertoli cells were present, suggesting that either of these cell types was the site of origin of the FSH inhibitory substance.

Testicular extracts were found to contain a proteinaceous subject, named inhibin, that was capable of suppressing FSH release either *in vivo* or *in vitro* (Franchimont *et al.*, 1978, Davies *et al.*, 1978). Isolated Sertoli cells were found to release a substance with similar FSH-suppressing properties into the culture medium (Steinberger & Steinberger, 1976).

Inhibin has been characterised as a gonadal glycoprotein that preferentially suppresses FSH secretion (Burger & Igarashi, 1988), by reducing FSH β mRNA concentrations (Attardi *et al.*, 1989) and blocking GnRH stimulated LH and FSH release (Kotsuji *et al.*, 1988). Leydig cells also secrete immunoactive inhibin (Risbridger *et al.*, 1989), though the contribution that Leydig cells make to inhibin levels in the testis and periphery is unclear, as Maddocks & Sharpe (1989) demonstrated that

destruction of Leydig cells by EDS had no effect on the levels of immunoactive inhibin in serum or interstitial fluid.

Measurement of inhibin levels in peripheral and spermatic vein serum in men with varicocele-associated oligospermia has shown that inhibin is released from the human testis in well-defined pulses that coincide with episodes of testosterone release. As FSH pulses do not coincide with LH pulses, the association of inhibin and testosterone pulses implies that LH may have an important role in the regulation of inhibin release in men (Winters, 1990). This may be related to the Leydig cell being one source of immunoactive inhibin.

An antiserum to the α subunit of inhibin has been used to immunoneutralise inhibin in peripheral blood. In the immature male rat inhibin immunoneutralisation causes dramatic increases in plasma FSH concentrations, while inhibin immunoneutralisation in the adult had no effect on gonadotrophin secretion (Culler & Negro-Vilar, 1988). Following a series of experiments that examined the effects of both inhibin immunoneutralisation and selective removal of Leydig cells with EDS, Culler (1990) proposed that the suppressive effect of the testes on LH and FSH secretion was mainly due to the secretion of a Leydig cell factor, presumably testosterone.

2.3. Structure of the testis

2.3.1. Interstitial Tissue

The loose connective tissue found between seminiferous tubules contains Leydig cells, endothelial elements, lymphatics, small nerves, macrophages and blood vessels. The connective tissue stroma, consisting of a ground substance interlaced with collagen fibres and fibroblasts, supports the cells within the interstitium (Christensen, 1975, Kerr, 1989).

Leydig cells are a heterogeneous population of cells, located either singly or in clusters in the intertubular tissue. The interstitium provides a unique environment in which Leydig cells synthesize and secrete testosterone into the peripheral blood supply and the seminiferous

tubules. There are several recognised patterns of organisation of the testicular interstitium. For example, electron micrographs of perfused rat testis, show a sparse interstitium with a relatively small volume of Leydig cells (1-5% of the total testicular volume) and a minimum of connective tissue. The majority of the intertubular area is occupied by peritubular lymphatic sinusoids. In contrast, in the human testis the interstitium consists of random clusters of Leydig cells scattered in very loose, fluid-filled, connective tissue. Leydig cells comprise between 5-12% of the total testicular volume (Fawcett *et al.*, 1973, Christensen, 1975, Kaler & Neaves, 1978). Clusters of Leydig cells are connected by numerous gap junctions, and these are considered to be a site of direct intercellular communication, and are perhaps important in coordinating steroid synthesis (Schulze, 1984).

2.3.1.1. Leydig cell structure and heterogeneity

Leydig cells are polygonal and have a diameter of 15-20μM. Their prominent nucleus is round or oval and has one or more prominent nucleoli. Leydig cells contain organelles which reflect their function as steroid producing cells. The most abundant organelle is the smooth endoplasmic reticulum (SER). This is composed of interconnected membrane tubules which form a continuous network throughout the cytoplasm. Scattered patches of rough endoplasmic reticulum (RER) interconnect with the SER. Mitochondria are found throughout the cytoplasm. A Golgi complex is usually found at one pole of the nucleus and centrioles are usually in the Golgi region. The cytoplasm also contains lipid droplets, microtubules and microfilaments, lysosomes and vacuoles. In the human, Leydig cell cytoplasm may also contain rod-shaped crystals of Reinke. These are proteinaecous and found in various sizes and numbers. Reinke crystals are not necessarily present and their function is unknown. Leydig cells also contain peroxisomes. These are granules of approximately 0.2μm diameter, bounded by a single membrane, which contain several oxidases and a catalase. Their function in Leydig cells is unclear. (Christensen, 1975, Schulze, 1984, Kerr, 1989). However, Leydig cell peroxisomes contain sterol carrier protein-2 (SCP_2) which participates in the intracellular transport of cholesterol to

mitochondria. Hence peroxisomes may use SCP_2 to provide cholesterol for conversion to testosterone (Mendis-Handagama *et al.*, 1990).

Most of the enzymes involved in producing testosterone are located in the SER. Thus, the abundance of SER provides a large surface area for steroidogenic reactions. It has been estimated that the total membrane surface area of SER in a human Leydig cell is $4000\mu m^2$. The SER in the rat is even more abundant, with a total membrane surface area two or more times greater (Christensen, 1975).

The capacity of Leydig cells to produce testosterone is strongly correlated with the volume of Leydig cell SER. The ability of a perfused testis to produce testosterone in response to LH was related to the mean volume density of SER, as assessed at the electron microscopic level in the contralateral testis. These two factors were found to be linearly and positively correlated in 5 species ($r=0.99$ in rat, rabbit, guinea pig, dog and hamster, Zirkin *et al.*, 1980).

Leydig cell lipid droplets contain predominantly cholesterol esters and neutral fats. The esters can be released into the cytoplasm where they are hydrolysed by soluble esterases to provide free cholesterol for steroid synthesis (Christensen, 1975, Schulze, 1984, Ewing & Zirkin, 1983). In a population of human Leydig cells the size and number of lipid droplets varies between cells. As the droplets contain precursors for steroid synthesis, it has been suggested that cells with fewer or smaller droplets are more steroidogenically active than those with more droplets. In agreement with this, the number of lipid droplets decreases after LH stimulation and increases temporally following hypophysectomy (Christensen, 1975). The variation in lipid droplets from cell to cell has been interpreted as evidence for a Leydig cell secretory cycle. In fact, the heterogeneity of Leydig cells in both rat and human testes is a well documented phenomenon. In the human, Schulze (1984) described a heterogeneous population of Leydig cells, which are variably stained (light versus dark) with toluidine blue. One of the issues addressed in this thesis is whether light and dark Leydig cells can be distinguished on morphological (size, distribution) or functional (steroidogenic capacity) grounds.

The heterogeneity of Leydig cells is illustrated by the separation of populations of different densities by density gradient centrifugation. This

is the case for both rat (Payne *et al.*, 1980, Cooke *et al.*, 1981) and human (Papadopoulos *et al.*, 1987a, Simpson *et al.*, 1987) Leydig cells. Papadopoulos *et al.* (1987a) described 2 populations of human Leydig cells with densities between 1.045-1.055g/ml and 1.075g/ml. The Leydig cells in the first group had higher basal levels of cAMP and testosterone than those in the second group but were less responsive to hCG. Simpson *et al.* (1987) also demonstrated that human Leydig cells can be separated on the basis of density, but not at the same densities as Papadopoulos *et al.* (1987a). Further, Simpson *et al.* (1987) found no difference in the steroidogenic capacity of Leydig cells of different densities.

Payne *et al.* (1980) separated Leydig cells on metrizamide gradients and found two populations of differing densities that had equal ability to bind ^{125}I-hCG. However, one population showed a much lower response to hCG or cAMP. The authors suggested that this population was composed of immature Leydig cells that had the ability to bind hCG but no capacity to respond. The results of this study may be open to other interpretations as it is known that metrizamide can enter cells. However, a second study which used Percoll gradients gave very similar results. Cooke *et al.* (1981) used Percoll to isolate Leydig cells with a range of densities, and a range in their responsiveness to hCG. All Leydig cells were able to bind hCG. The Leydig cells with the highest testosterone output were found to comprise only 6% of the total population, indicating that in the rat testis at any one time there are a large number of Leydig cells which have a low response to hCG. The authors suggested that in the rat, Leydig cells may be undergoing a continuous cycle of metabolic activity. This hypothesis is supported by the work of Bergh (1983, 1985), who showed that when the testosterone requirement of seminiferous tubules is at its peak at stage VII, adjacent Leydig cells are larger than the Leydig cells adjacent to tubules at other, less testosterone dependent stages. Using tissue obtained after death, Regadera *et al.* (1991) demonstrated that human Leydig cells do not show a uniform ability to bind an anti-testosterone antibody. The degree of staining in Leydig cells ranged from very intense to none at all. Again, such differences may be due to cyclical changes in the functional activity of Leydig cells.

As all Leydig cells within the interstitium are exposed to equivalent amounts of circulating gonadotrophins, the hypothesis of a

Leydig cell cycle must necessarily imply regulation by locally produced factors. This will be discussed in more detail in section 2.4.

2.3.1.2. Leydig cell steroidogenesis

The primary precursor of androgen synthesis is the 27-carbon sterol, cholesterol, which can be obtained from three separate sources. The main source is *de novo* synthesis from acetate, which occurs in the SER. Leydig cell lipid droplets contain cholesterol esters which can be hydrolysed to cholesterol by plasma esterases. Finally, cholesterol can be obtained directly from the circulating blood supply. It is probably transported to the Leydig cell by lipoproteins which are thought to interact with membrane lipid receptors (Ewing & Zirkin, 1983, Rommerts & van der Molen, 1989).

Cholesterol is transported to the mitochondria where it is converted to pregnenolone by removal of the 6-carbon side chain on the C-17 of cholesterol. The reaction is catalysed by a P450-dependent side chain cleavage enzyme, $P450_{scc}$, which is regulated by LH. Pregnenolone can be converted to testosterone through either the Δ^4 or Δ^5 pathway. The difference refers to the position of the unsaturated bond in the intermediates formed in the conversion process. In the Δ^4 pathway, the unsaturated bond is between C-4 and C-5 of ring A, and in the Δ^5 pathway it is between C-5 and C-6 of ring B. The rate of conversion of testosterone via each pathway is different in different species. In the rat the Δ^4 pathway predominates, while the Δ^5 is the preferred route of testosterone synthesis in the human testis.

The conversion of pregnenolone occurs in the SER. In the Δ^4 pathway the intermediates, progesterone, 17-hydroxyprogesterone and androstenedione are formed by the actions of 3β-hydroxysteroid dehydrogenase (3βHSD), P450 17α-hydroxylase and C_{17-20} lyase respectively. In the Δ^5 pathway the latter two enzymes are responsible for the formation of 17-hydroxypregnenolone and dehydroepiandrosterone. Androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD). Dehydroepiandrosterone is converted to testosterone by prior conversion to either androstenedione or androstenediol (for review see Preslock, 1980, Rommerts & van der Molen, 1989).

A decrease in activity after hypophysectomy in the adult rat demonstrates that steroidogenic enzymes require the continued presence of LH for their activity. Administration of hCG reverses the inhibitory effects of hypophysectomy (Purvis *et al.*, 1981, Rommerts & van der Molen, 1989 for review). The acute stimulatory effect of LH is to increase the rate of transport of cholesterol into the inner mitochondrial membrane where it associates with $P450_{scc}$. Chronic stimulation by LH is required for optimal synthesis of P450 enzymes, though the mechanism by which LH (and cAMP) regulates transcription of the P450 genes has not been established (Payne, 1990). In bovine adrenocortical (John *et al.*, 1986) and MA-10 (Hales *et al.*, 1990) cell cultures cAMP is able to induce increases in the steady state levels of transcription of $P450_{scc}$ and P450 17α-hydroxylase mRNAs. These increases have been shown to reflect increases in the rate of gene transcription.

Keeney & Mason (1992) have shown that the LH-dependent expression of 3βHSD is regulated via a cAMP-dependent signalling pathway. Positive regulation by LH, dibutyryl cAMP, forskolin and cholera toxin was seen at all stages from the transcription of the 3βHSD gene to translation of the mRNA and formation of a functional enzyme.

Testosterone appears to be secreted immediately after its synthesis. The process does not seem to involve secretory vesicles, as none have been observed in Leydig cells. Also, the rate of testosterone secretion correlates highly with the intratesticular testosterone content (Eik-Nes, 1971). Testosterone probably enters target cells by passive diffusion. Three possible fates await testosterone in the various target cells it reaches (Wilson, 1992). It can combine with the androgen receptor to form a hormone-receptor complex in the nucleus, it may be aromatised to dihydrotestosterone which has a higher affinity for the androgen receptor, or it may be aromatised to oestrogen and mediate effects through the oestrogen receptor.

The androgen receptor belongs to a family of gene regulatory proteins, which are characterised by their organisation into functional domains. The receptor has sites for specific, high affinity ligand binding, for DNA-binding to the steroid response element of the target gene and for transcriptional modulation (O'Malley & Tsai, 1992). In the rat testis, androgen receptors have been localised to Sertoli cells (Grootegood *et al.*,

1977), Leydig cells and peritubular cells (Sar *et al.*, 1990). The presence of androgen receptors on Leydig cells implies the existence of a short loop negative feedback effect of testosterone on its own production. This was demonstrated by Darney & Ewing (1981), who infused testosterone directly into a perfused rat testis and found that testosterone levels in the perfusate fell to 50% of the control within 20 minutes. A similar control system probably exists in man, as androgen receptors have been localised to Leydig cells using immunohistochemical techniques (Sar *et al.*, 1990) and androgen receptor mRNA has been found in purified human Leydig cells (Namiki *et al.*, 1991). However, testicular arterioles also have androgen receptors (Bergh, 1991) therefore the possibility exists that infusion of testosterone might, through an action at these receptors, decrease testicular blood flow, thereby reducing the entry of LH to the testis and the escape of testosterone from it. This could also account for the reported decrease of testosterone levels in the perfusate.

2.3.1.3. Other Leydig cell products

The major function of Leydig cells is the production of androgen. Indeed, as the chemical removal of Leydig cells with EDS in conjunction with simultaneous administration of exogenous testosterone, can maintain quantitatively normal spermatogenesis, the importance of Leydig cell products other than testosterone is not obvious (Ratnasooriya & Sharpe, 1989). However, as there is increasing evidence for the existence of local, cell to cell, regulatory mechanisms in the testis, the importance of other Leydig cell products in maintaining optimal conditions for spermatogenesis may be clarified.

Oestradiol The formation of oestradiol from testosterone requires the action of an aromatase enzyme. Antibodies directed against this enzyme have been used to demonstrate its existence in immature and adult rat Leydig cells. No other cell type contained the enzyme, hence the Leydig cell is the site of oestradiol synthesis in the adult rat testis (Kurosumi *et al.*, 1985). Administration of oestrogen suppresses plasma testosterone levels in rat and man. This was thought to be solely due to an effect on pituitary gonadotrophins (see Purvis & Hansson, 1978 for review).

However, Bartke *et al.* (1977) demonstrated that addition of oestrogen to testicular cultures significantly inhibited the accumulation of testosterone into the culture medium. Also, the administration of oestrogen in rat and man results in a drop in testosterone levels before there is a detectable change in LH levels (Purvis & Hansson, 1978). Oestrogen receptors have been localised to rat Leydig cells (Mulder *et al.*, 1973), though one study failed to detect binding of an anti-oestrogen receptor antibody to human Leydig cells (Due *et al.*, 1989). Thus intratesticular oestrogens, like testosterone, could exert a direct regulatory effect on testosterone synthesis. Monitoring changes in the levels of steroid precursors following incubation of testicular homogenates with oestrogen demonstrated that the primary effect of oestrogen was to decrease the activity of $C_{17\text{-}20}$ lyase (Kalla *et al.*, 1980). This effect of oestrogen has also been observed in microsomes prepared from neonatal pig testis (Onoda & Hall, 1981). It is possible that oestrogen has a direct inhibitory effect on the P450 enzyme as the oestrogenic suppression of testosterone levels is not blocked by the anti-oestrogen, tamoxifen (Damber *et al.*, 1983).

Oxytocin Immunoreactive oxytocin-like material has been detected in both human and rat testes (Nicholson *et al.*, 1984), and has been specifically localised to Leydig cells (Guldenaar & Pickering, 1985). Isolation of oxytocin mRNA from bovine Leydig cells confirms that Leydig cells are the testicular source of oxytocin (Ivell & Burbach, 1991).

The effects of oxytocin on Leydig cells are unclear. One study demonstrated that oxytocin had an inhibitory effect on testosterone production by isolated mouse Leydig cells (Tahri-Joutei & Pointis, 1988). However, no effect of oxytocin on cultures of purified rat Leydig cells has been demonstrated (Sharpe & Cooper, 1987).

Oxytocin appears to have a role in controlling the contractions of the myoid cells which surround seminiferous tubules. These contractions may be responsible in part for the transportation of spermatozoa to the rete testis (Wathes, 1984).

POMC-peptides Testicular pro-opiomelanocortin derived peptides include ACTH, α-MSH and β-endorphin. Their production by Leydig cells has been demonstrated in rat and man (Bardin *et al.*, 1984). POMC mRNA

has been localised to Leydig cells in the adult rat testis (Pintar *et al.*, 1984). However, the removal of Leydig cells from the rat testes following administration of EDS, did not result in changes in the levels of total testicular POM-C mRNA, nor of POM-C peptides (Li *et al.*, 1989). The authors were also unable to detect ACTH or β-endorphin in conditioned media from a purified adult rat Leydig cell preparation, suggesting that Leydig cells are not an important source of POM-C peptides *in vivo*. However, Eskeland *et al.* (1992) were able to measure β-endorphin production by adult rat Leydig cells *in vitro*, and also demonstrated that CRF was able to stimulate the release of β-endorphin.

Opiate receptors have been localised to Sertoli cells (Fabbri *et al.*, 1988) and β-endorphin has been shown to inhibit FSH-induced cAMP accumulation. In young rats β-endorphin also inhibits ABP production, but has no effect on ABP secretion in adult rats (Fabbri *et al.*, 1988, Gerandi *et al.*, 1984). In contrast, other POMC peptides such as ACTH and α-MSH stimulate Sertoli cell cAMP formation (see Heindel & Treinen, 1989 for review). The secretion of β-endorphin from cultured mouse Leydig cells is under hormonal control; GnRH inhibits β-endorphin production while hCG had a stimulatory effect (Fabbri & Dufau, 1988).

Inhibin In the testis the major site of production of inhibin is the Sertoli cell (for review see Ying, 1988). However, immunoreactive and bioactive inhibin have been detected in the culture medium of Leydig cells from adult rats (Risbridger *et al.*, 1989). A separate study found that Leydig cells were only able to produce immunoactive inhibin (de Winter *et al.*, 1992). Precipitation of ^{35}S-methionine labelled proteins revealed that mature rat Leydig cells secrete a 44kDa inhibin-related protein, possibly proαNαC (de Winter *et al.*, 1992). It has been reported that administration of hCG increases serum levels of immunoactive inhibin in adult rats (Sharpe *et al.*, 1988) and in man (McLachlan *et al.*, 1988). However the destruction of Leydig cells by EDS does not lead to any decrease in the levels of immunoactive inhibin in blood or testis (Maddocks & Sharpe, 1989). The contribution that Leydig cells make to the levels of inhibin in the testis and periphery requires further study.

2.3.1.4. Macrophages

Macrophages are common in interstitial tissue and may be associated with Leydig cell clusters. In the rat, macrophages are believed to constitute approximately 25% of the cells in the interstitium (Niemi *et al.*, 1986). Conditioned medium from cultured rat macrophages can stimulate basal and LH-stimulated testosterone production by purified Leydig cells. Macrophages that had been cultured in the presence of FSH were more able to stimulate testosterone production than macrophages that had been cultured under control conditions (Yee & Hutson, 1985). However, neither of these findings have been confirmed independently. Sertoli cells secrete interleukin-1α, which may regulate some functions of macrophages (Bellve & Zheng, 1989). If testicular macrophages are influenced by Sertoli cells and can themselves affect Leydig cell function, then testicular macrophages may have an important role in coordinating testicular function. Evidence exists to support this hypothesis; interleukin-1β (IL-1β) is one of the major cytokines released by macrophages (Dinarello, 1991) and IL-1β has direct effects on isolated Leydig cells. However, there is some disagreement over the nature of the effects that IL-1β has on Leydig cell function. IL-1β has been reported to inhibit hCG and cAMP-stimulated testosterone formation in primary cultures of Leydig cells (Calkins *et al.*, 1990), and to inhibit $P450_{scc}$ mRNA expression in isolated adult rat Leydig cells (Lin *et al.*, 1991). In contrast, Warren *et al.* (1990) demonstrated that IL-1β was able to stimulate the basal secretion of testosterone and to augment maximally hCG-stimulated testosterone production.

2.3.2. Seminiferous Epithelium

The epithelium of each adult seminiferous tubule is composed of two populations of cells. There are the non-proliferating, immobile supporting elements, the Sertoli cells, plus a mobile population of dividing and differentiating germ cells (Schulze, 1984, Trainer, 1987).

There is a close morphological relationship between Sertoli cells and developing germ cells. The cytoplasm of each Sertoli cell extends from the basal lamina to the tubule lumen and sends out processes which

surround germ cells, so that every germ cell is contacted and supported by a number of adjacent Sertoli cells. In this way the Sertoli cell creates a lattice-like network of cellular extensions and processes within which germ cells proceed through the stages of spermatogenesis. Hence the Sertoli cell is in contact with all generations of germ cells at the same time (Schulze, 1984, Tindall *et al.*, 1985).

Developing germ cells are found in characteristic associations throughout the seminiferous epithelium. In the rat, germ cells are arranged in a regular pattern, or stage, which often occupies the complete tubular cross-section. Stages are arranged mostly in serial continuity along the length of a tubule. In the rat 14 germ cell associations or stages are recognised, according to the characteristics of the acrosome of the spermatids as seen by periodic acid-Schiff staining (Leblond & Clermont, 1952). In man, the arrangement of germ cell stages has been deduced by following the spatial position and degree of development of various populations of primary spermatocytes. In this way it has been shown that in the human testis the developmental sequence of germ cells is arranged along a spiral (Schulze & Rehder, 1984). The close morphological association of Sertoli cells and germ cells implies that the Sertoli cell plays an important role in spermatogenesis.

2.3.2.1. Organisation of spermatogenesis

There are four basic germ cell types; spermatogonia, primary and secondary spermatocytes and spermatids, and three main phases of spermatogenesis; spermatogonial multiplication, meiosis and spermiogenesis.

Spermatogonia are large diploid cells which arise from the gonocytes of the fetal testes. Morphologically, A, intermediate and B type spermatogonia can be distinguished. They undergo several mitoses (usually 5 or 6) before entering the first stage of meiosis, when they are classified as preleptotene spermatocytes. In this stage the chromosomes begin to spiral and contract. The next developmental step is the formation of zygotene spermatocytes, which are characterised by the pairing off of analogous chromosomes, which begin to thicken as they gather together. This is followed by the pachytene stage, the longest meiotic step.

Pachytene spermatocytes are distinguished by thickened chromosomes which are held together only at the centromere. As the nucleus reaches its maximal size, duplication is complete and tetraploid diplotene spermatocytes are formed. These undergo division to give rise to two secondary (diploid) spermatocytes, which in turn undergo a further mitotic division to form haploid cells. Spermiogenesis is the process by which mature elongate spermatids are formed. Its regulation is not understood but it involves nuclear condensation, acrosome formation, the development of a tail and the arrangement of mitochondria into a helix to form the midpiece of the spermatozoon (Roosen-Runge, 1962). In the rat, one wave of spermatogenesis, i.e. the formation of a mature spermatid from a type A spermatogonium, takes 65 days (Clermont & Harvey, 1965). In man, the time taken for total germ cell maturation is 70±4 days, although spermatogenesis proceeds along the same pathway as that described for spermatogenesis in the rat (for review see Trainer, 1987).

2.3.2.2. Sertoli Cells

Sertoli cells are tall, columnar cells which extend from the basal lamina to the lumen of the seminiferous tubule. They have a characteristic, highly lobulated nucleus with prominent nucleoli. The nucleus is generally found in the second layer of the epithelium, that is, above spermatogonia. Sertoli cell cytoplasm contains mitochondria, Golgi apparatus, abundant SER, also RER and a three-dimensional network of intermediate filaments. The Sertoli cell cytoskeleton also contains microfilaments and microtubules. At least one cytoskeletal element is believed to be involved in every cellular process. A feature of Sertoli cell cytoplasm is that SER is often found in concentric aggregations around lipid droplets or lysosomes (Schulze, 1984, Tindall *et al.*, 1985).

2.3.2.3. Formation of the blood-testis barrier

Close to the base of the seminiferous epithelium, contact surfaces of adjacent Sertoli cells show symmetrically developed specialised junctions; these form what is known as the blood-testis-barrier. This is a

permeability barrier around seminiferous tubules which was discovered when proteins that were abundant in plasma and testicular lymph were found only in very low concentrations in rete testis fluid (Setchell, 1967). Dym & Fawcett (1970) demonstrated that when lanthanum, a small molecular weight, electron-opaque substance, was introduced into the testis, it was able to permeate the space between spermatogonia and surrounding germ cells, but was prevented from going deeper into the epithelium by focal tight junctions on the interfaces between adjacent Sertoli cells. The authors described how pairs of Sertoli cell are joined above the spermatogonia resting on the basal lamina, but below the spermatocytes. These Sertoli cell junctions divide the epithelium into a basal and an adluminal compartment. The basal compartment therefore contains the spermatogonia and preleptotene spermatocytes, while the adluminal compartment contains late spermatocytes and developing spermatids. The pattern of Sertoli cell junctions must be modified at certain stages of the spermatogenic cycle to allow preleptotene spermatocytes to move from the basal to the adluminal compartment.

There are two functional components to the blood-testis-barrier. The first is the peritubular layer of myoid cells in the boundary tissue of the seminiferous tubule. Intercellular junctions between adjacent myoid cells which prevent several substances from entering a seminiferous tubule. The morphological site of the blood-testis barrier is the series of junctional specialisations between adjacent Sertoli cells. There are three different types of junctions between Sertoli cells. There are zones of close contact and also desmosome-like junctions which have an adhesive-like capacity to connect adjacent cells. They are also found between Sertoli cell and spermatogonia and spermatocytes. The most common connection made between two Sertoli cells is the tight junction. These consist of focal fusions of the outer leaflets of opposing cell membranes. One constituent of Sertoli cell junctional complexes is actin. In other non-muscle cells actin is controlled by calcium which regulates a relaxation-contraction cycle. It has been hypothesised that free calcium released from the endoplasmic reticulum could become bound to calmodulin and promote the motile force of the microfilaments (Means *et al.*, 1980). Junctional specialisations are also found between Sertoli cells facing germ cells. It is

therefore possible that active changes in Sertoli cell shape may affect the movement of germ cells from the base to the lumen of the tubule.

The existence of the blood-testis barrier means that blood-borne nutrients and gonadotrophins which have successfully crossed the peritubular myoid layer, have direct access to spermatogonia and preleptotene spermatocytes, but to reach later germ cells must pass through Sertoli cell cytoplasm. A further consequence of the formation of Sertoli cell tight junctions is the polar differentiation of Sertoli cell cytoplasm. Basal cytoplasm contains an abundance of organelles and inclusions, while apical cytoplasm contains only axially oriented mitochondria, cisternae of the SER, microtubules and filaments, and occasionally, glycogen. For these reasons it is believed that the function of the blood-testis barrier is to isolate late spermatocytes and early spermatids in order to provide the optimal environment as they undergo meiosis and spermiogenesis (Dym & Fawcett, 1970, Schulze, 1984, Tindall *et al.*, 1985).

2.3.2.4. Sertoli cell functions

Specific functions ascribed to Sertoli cells include fluid production, phagocytosis, maturation and release of spermatozoa and protein synthesis. Seminiferous tubule fluid (STF) has higher concentrations of potassium and bicarbonate than are found in plasma, and lower concentrations of sodium and chloride. This gradient is maintained by the Sertoli cell tight junctions and Sertoli cell cytoplasm (Setchell, 1974)

Sertoli cells have pronounced endocytic activity, phagocytosing both degenerating germ cells and the residual cytoplasm left behind when mature spermatozoa are released (Black, 1971). The nature of the Sertoli cell factors required for maturation of spermatids remains unclear. Recently, it has been proposed that dialogue between Sertoli cells and residual bodies is important in paracrine regulation of this process. According to the hypothesis proposed by Jegou *et al.* (1992), residual bodies may regulate Sertoli cell function by two pathways. The mRNA released by Sertoli cell phagocytosis of the residual body may code for a factor(s) which controls Sertoli cell gene expression. The expressed Sertoli cell proteins may then regulate different events at the germ cell level. The

actual process of phagocytosis of residual bodies may trigger the production of a factor(s) able to regulate Sertoli or germ cell function. A postulated mechanism for the release of the mature spermatozoa, or spermiation involves a physiological change in Sertoli cell junctional specialisations resulting in the loss of adhesion and the consequent release of the sperm head from its attachment site (Ross, 1976).

2.3.2.5. Synthesis and secretion of Sertoli cell proteins

An important function of Sertoli cells is the active secretion of proteins. Cultured Sertoli cells will incorporate ^{35}S-methionine into newly synthesized proteins that are then released into the culture medium. The secreted radiolabelled proteins can be visualised by 2-dimensional gel electrophoresis. Using this technique, Sertoli cells *in vitro* have been found to secrete over a hundred different proteins (Wright *et al.*, 1983, Sharpe *et al.*, 1992). As very few secretory vesicles have been seen in Sertoli cells either *in vivo* or *in vitro*, it appears that the proteins secreted by Sertoli cells are needed immediately by the surrounding germ cells (Tindall *et al.*, 1985). The rate of production of Sertoli cell proteins *in vitro* can be assessed by measuring the rate of incorporation of ^{3}H-leucine. Using this technique, Rommerts *et al.* (1978) demonstrated that FSH, cAMP and testosterone were all able to stimulate the secretion of labelled proteins from cultured immature Sertoli cells.

Sertoli cells secrete their products in a bidirectional manner. Substances can either be secreted via the apex of the Sertoli cell into STF, or via the base of the cell into interstitial fluid, and from there into the peripheral blood supply. Unidirectional secretion has not been demonstrated for any Sertoli cell product, yet the secretion of many is known to be predominantly in one direction (for review see Sharpe, 1988). Bicameral chambers enable the study *in vitro* of the polarized secretion of proteins by cultured Sertoli cells. This system has been used to demonstrate bidirectional secretion of radiolabelled Sertoli cell proteins *in vitro* (Djakiew & Dym, 1988).

Radiolabelled proteins secreted by segments of seminiferous tubules cut into distinct spermatogenic stages can be visualised by 2-dimensional gel electrophoresis. This technique has been used to

illustrate how the pattern of proteins secreted varies from one spermatogenic stage to the next, with the secretion of at least 15 different proteins following a cyclical pattern (Wright, 1983, McKinnell *et al.*, 1992). The ability of Sertoli cells to secrete proteins in a directed and cyclical manner and the significance of this to regulation of spermatogenesis will be discussed with reference to specific Sertoli cell proteins.

Androgen binding protein (ABP) ABP is a 105kDa protein composed of two subunits. It shows high affinity binding for testosterone and dihydrotestosterone. It is secreted into STF then through the rete testis and efferent ducts into the epididymis (for review see Tindall *et al.*, 1985). Results of a series of experiments have confirmed that Sertoli cells are the site of ABP synthesis. Androgen binding capacity was measured in interstitial and tubular tissue and found to be maximal in tubular tissue; destruction of germ cells by irradiation was found not to affect testicular ABP levels (see Hansson *et al.*, 1976 for review). Most conclusively, immature rat Sertoli cell cultures produce ABP *in vitro*. Cultures of human Sertoli cells also produce an androgen binding activity which co-elutes with rat ABP (Lipshultz *et al.*, 1982). The rat testis contains a testis-specific form of ABP (French & Ritzen, 1973), but it is not clear if this is the case in man. Purvis *et al.* (1978) demonstrated that the androgen binding component of the human testis shared the same physicochemical properties as testosterone binding globulin (TeBG, or sex hormone binding globulin), which is found in the serum of man but not the rat. Cheng *et al.* (1985) isolated two molecular species of human testicular ABP. One form was very similar to TeBG, but the other was different from TeBG with regard to the molecular weight of its monomers, its carbohydrate structure and peptide sequence.

The secretion of ABP by Sertoli cells *in vitro* is stimulated by FSH, cAMP and testosterone (Fritz *et al.*, 1976, Rommerts *et al.*, 1978). In the rat approximately 80% of ABP is secreted into the tubule lumen, the remaining 20% is found in interstitial fluid (Mather *et al.*, 1983). However, it has been demonstrated that under conditions of impaired spermatogenesis, for example following testosterone withdrawal consequent to Leydig cell destruction or induction of unilateral cryptorchidism, the pattern of ABP secretion changes. In such situations,

although the rate of production and the total testicular content of ABP decrease, the amount of ABP secreted into interstitial fluid increases by as much as 200% (Sharpe & Bartlett, 1987 and Sharpe, 1988 for review). The physiological significance of such changes is unclear.

The secretion of ABP is stage-dependent. Ritzen *et al.* (1982) cultured stage dissected tubules from adult rats and found that ABP secretion into the culture medium was highest between stages VIII-XI and lowest between stages II-V. This coincides with the distribution of ABP mRNA measured in stage-synchronised rats (Parvinen, 1992 for review).

Gerard *et al.* (1991), demonstrated that ABP and TeBG are endocytosed by germ cells, suggesting that steroid binding proteins may act as transmembrane steroid carriers during spermatogenesis.

Inhibin and Activin Inhibin can selectively suppress FSH synthesis and release from gonadotrophs in the anterior pituitary. Its production by Sertoli cells was demonstrated by Steinberger & Steinberger (1976). Co-culture of anterior pituitary cells and immature Sertoli cells, or the addition of Sertoli cell conditioned medium to cultured anterior pituitary cells resulted in a significant decrease in the levels of FSH in the culture medium.

Inhibin exists as a heterodimer of α and β (either β_A or β_B) subunits, linked by disulphide bridges. Thus two forms of inhibin are recognised on the basis of the form of the β subunit which they contain, namely inhibin-A and inhibin-B. Activin exists as a homodimer of inhibin β subunits, activin A ($\beta_A\beta_A$) and activin AB ($\beta_A\beta_B$) forms are recognised (see Ying, 1988, de Kretser & Robertson, 1989 for review). The mRNA for the α, β_A and β_B subunits of inhibin has been localised to Sertoli cells (Toebosch *et al.*, 1988). In Sertoli cells the production of inhibin is stimulated by the actions of FSH, cAMP analogues and phosphodiesterase inhibitors, suggesting that FSH stimulates inhibin production via a cAMP-dependent mechanism (de Kretser & Robertson, 1989).

The expression of inhibin α and β_B subunit mRNA is cyclical. The highest expression occurs in stages XIII-I, and the lowest level of expression is seen in stages VII-VIII. The secretion of immunoreactive inhibin follows the same pattern (for review see Parvinen, 1992). By using

flow cytometry to selectively analyse specific populations of rat spermatocytes in culture, Woodruff *et al.* (1992) demonstrated that inhibin-A binds to several subpopulations of germ cells. The implication is that spermatogonia, spermatocytes and early spermatids have specific receptors through which inhibin-A can exert direct effects. In fact, inhibin has been postulated to have a role in regulating spermatogonial numbers (van-Dissel-Emiliani *et al.*, 1989). Inhibin purified from rat Sertoli cell conditioned medium was found to decrease the numbers of differentiating spermatogonia in adult mice testes, with spermatogonia in stages I-VI being most sensitive to the effects of inhibin.

Activin stimulates the secretion of FSH by pituitary cells *in vitro* (Ying, 1988). Activin may also have a role in mediating Sertoli cell-Leydig cell interactions. Lin *et al.* (1989) demonstrated an inhibitory effect of activin on hCG-stimulated cAMP and testosterone formation in cultured adult rat Leydig cells. Using cultures of immature porcine Leydig cells, Mauduit *et al.* (1991) found that activin A inhibited hCG-stimulated dehydroepiandrosterone accumulation, possibly via partial inhibition of $P450_{scc}$.

Transferrin Transferrin is a single glycoprotein with an M_r of 76.5kDa. It probably functions as an iron transport protein. Sertoli cells *in vitro* secrete transferrin (Skinner & Griswold, 1980). Measurement by radioimmunoassay has shown that transferrin levels in STF and rete testis fluid are much lower than the levels found in serum and lymph, suggesting that Sertoli cell transferrin is secreted basally (Sylvester & Griswold, 1984), although transferrin is also produced by the liver and secreted into blood and lymph, and the study failed to distinguish between liver and Sertoli cell forms. In the same study, indirect immunofluoresence techniques were used to localise transferrin to the interstitium and also to the developing acrosome and nuclear cap of early spermatids. Early spermatids are able to bind and internalise transferrin by a receptor-mediated endocytotic process (Segretain *et al.*, 1992). Sertoli cell transferrin may thus serve as an intermediate in the transport of iron from serum transferrin to germ cells in the adluminal compartment of the seminiferous epithelium. In this context, it has been demonstrated that the addition of pachytene spermatocyte conditioned

medium to cultured Sertoli cells stimulates transferrin secretion (Djakiew & Dym, 1988).

Quantification of silver grains in *in situ* hybridisation studies demonstrated that while a transferrin RNA probe bound exclusively to Sertoli cell sequences, the levels of mRNA were highest in stages XIII-XIV and fell at stage VIII to a low at stage IX (Morales *et al.*, 1987). This coincides with the study by Wright *et al.* (1983) who showed that transferrin secretion was maximal at stages XIII-XIV and lowest at stages VII-VIII. The expression of transferrin mRNA by Sertoli cells *in vitro* is hormonally regulated, being stimulated by FSH in combination with insulin, retinol and testosterone. FSH alone had minimal effects on mRNA levels (Huggenvik *et al.*, 1987).

Sulphated-Glycoprotein-2 (SGP-2) SGP-2 consists of two subunits of 37 and 47kDa, linked by disulphide bonds (Collard & Griswold, 1987). Localisation by *in situ* hybridisation has shown that Sertoli cells are the only testicular cells which express SGP-2 mRNA (Morales *et al.*, 1987). Immunofluoresence techniques have localised secreted SGP-2 to the acrosome and distal tail portion of mature spermatozoa (Sylvester *et al.*, 1984).

The function of SGP-2 in the testis is unclear, it may be involved in lipid transport/metabolism on the sperm surface membrane (Sylvester *et al.*, 1991). Alternatively, SGP-2 has been shown to be similar to a serum protein which is a potent inhibitor of terminal complement complexes. The complement system consists of over 20 plasma proteins that together form a macromolecular protein complex that causes lysis of cell membranes on target cells such as bacteria and viruses. The localisation of SGP-2 on the surface of sperm, and its function as a complement inhibitor has lead to a proposed role for SGP-2 in preventing the complement mediated immobilisation of sperm cells in the female reproductive tract (Jenne & Tschopp, 1989).

In support of this hypothesis, the highest levels of immunoreactive SGP-2 are found at stages VII-VIII, before spermiation, and the lowest levels are found immediately after spermiation at stages XIII-I (Kangasniemi *et al.*, 1992). However, 2D-SDS PAGE analysis of radiolabelled proteins secreted by seminiferous tubules *in vitro*, does not

show any differences in the amounts of SGP-2 secreted by seminiferous tubules grouped in stages II-V, VI-VIII or IX-I (McKinnell & Sharpe, 1992).

2.4. Paracrine control of the testis

2.4.1. Morphological evidence for local regulation of testicular function

The mammalian testis is divided into tubular and interstitial compartments, with the tubular compartment being further divided into basal and adluminal sections. Spermatogenesis takes place in the tubular compartment and is a highly organised and complex process, throughout which there is an intimate association between Sertoli cells and all generations of developing germ cells.

The complexity of the spermatogenic process together with the structural arrangement of the testis points to an active interaction between different testicular cells. The regulation of testicular function is predominantly controlled by the action of pituitary gonadotrophins; hypophysectomy leads to testicular regression and malfunction. These effects are due to the absence of the hormone with the greatest influence on spermatogenesis, testosterone. Morphologically, gonadotrophin deprivation (for example due to hypophysectomy or the use of antisera to LH and FSH) is characterised by a marked increase in the rate of degeneration of mid-pachytene primary spermatocytes and step 7 and 19 spermatids, all of which are characteristically present in stage VII. These changes could be prevented by the administration of LH but not FSH. The same effects were caused by agents which blocked the action of androgens on Sertoli cells. Together such experiments indicate a special requirement for testosterone at stage VII (for review see Sharpe, 1983, 1992).

The first evidence indicating a role for local, cell to cell interactions in regulating testicular function also came from morphological studies. In 1978, Aoki & Fawcett implanted silastic capsules containing cyproterone acetate into one or both rat testes. The implants caused focal tubular involution, such that the tubules in the vicinity of the implants contained only Sertoli cells, being completely devoid of germ cells other

than spermatogonia. However, these effects were only apparent in tubules within a few millimetres radius of the implant. The remaining seminiferous tubules were unaffected. A further effect was the dramatic hypertrophy of interstitial tissue immediately adjacent to the affected tubules. In particular there was a large increase in the SER content of affected Leydig cells. Ewing & Zirkin (1983) showed that SER volume was directly related to steroidogenic output. Leydig cells adjacent to undamaged tubules were themselves unchanged. The authors concluded that the changes in volume and ultrastructure of Leydig cells could not be caused by an imbalance in feedback to the hypothalamo-pituitary axis, as any consequent elevation in serum gonadotrophins would cause a general stimulatory response in all of the Leydig cells, and not a specific local response in the area of the damaged seminiferous tubules. Thus, it was speculated that a diffusable product of the seminiferous epithelium may normally act on Leydig cells to modulate their sensitivity to LH, and damage to the tubules interfered with the production of this factor. Conversely, tubular damage might cause the release of a substance that has a direct stimulatory effect on Leydig cells.

Further evidence of direct interactions between seminiferous tubules and Leydig cells came from Risbridger *et al.* (1981). In the experimentally cryptorchid adult rat, Leydig cells show hypertrophy and hyper-responsiveness to hCG when cultured *in vitro*. A loss in ^{125}I-hCG binding is also seen *in vitro*. Experiments with unilaterally cryptorchid rats have shown that these changes cannot be due to elevations in serum LH levels. Leydig cells in the scrotal testis did not show the changes seen in Leydig cells in the abdominal testis despite being exposed to the same elevated levels of LH. Hence the changes in the abdominal testis must have been due to the actions of local factors.

Short term exposure of the testis to heat is accompanied by damage to the seminiferous epithelium (Chowdhury & Steinberger, 1964). Heat treatment also disrupts Sertoli cell function (assessed by decreasing ABP and STF production *in vitro*). The authors found that Sertoli cell function was not altered until between 2 and 4 weeks after exposure to heat. Thus, the impairment of Sertoli cell function is not a direct result of heating the Sertoli cell but was secondary to the immediate effects of heat on germ cell function. Restoration of spermatogenesis restored the measured

Sertoli cell parameters to normal. Leydig cell function was also altered, with Leydig cells becoming hypertrophic and showing increased responsiveness to hCG *in vitro*. Again, the changes in Leydig cell function were separated in time from the actual thermal stress. The alterations in Leydig cell function were therefore not due to the heat but may have resulted from specific germ cell changes either directly, or secondary to the disruption of Sertoli cells. Normal Leydig cell function was also restored as spermatogenesis recovered (Jegou *et al.*, 1984). In the course of this study serum LH and testosterone levels were not significantly altered at any stage.

These studies demonstrate that changes in testicular function occur independently of gonadotrophin action and thus support the hypothesis that cell to cell interactions have a vital role to play in supporting spermatogenesis.

2.4.2. Interactions between Sertoli cells and Leydig cells

Morphological evidence of a relationship between Sertoli cells and Leydig cells was provided by Bergh (1983, 1985), who demonstrated that the cell profile area of peritubular Leydig cells was dependent on the stage of the tubules the Leydig cells were closest to. Leydig cells were heterogeneous with respect to cell size, with the largest cells surrounding stage VII-VIII tubules and the smallest around tubules at stages IX-XIV. The probability that all these Leydig cells were exposed to equivalent amounts of LH, suggests that Leydig cell size is controlled at least partly via the seminiferous tubules. In a similar study in rats and monkeys, Fouquet (1987) found that in the rat, Leydig cells with the greatest intracellular volume of SER were associated primarily with stage VII-VIII tubules. However, in the monkey there was no relationship between either Leydig cell size or SER volume and the stage of the seminiferous epithelium.

Evidence from *in vitro* studies does support the hypothesis that Sertoli cells can modulate Leydig cell function. Co-cultures of Leydig cells with Sertoli cells, or the addition of Sertoli cell conditioned medium (SCCM) to Leydig cells *in vitro* stimulates steroid production. For example, Verhoeven & Cailleau (1985, 1986) demonstrated that SCCM prepared from cultures of immature Sertoli cells was able to stimulate

steroidogenesis in immature Leydig cells. Similar effects of SCCM have also been demonstrated with cells isolated from adult rats (Papadopoulos *et al.*, 1987b). Conditioned medium prepared from cultures of human Sertoli cells is able to stimulate testosterone production by isolated human Leydig cells (Papadopoulos, 1991). Leydig cell steroidogenesis is also stimulated by co-culture with Sertoli cells (Verhoeven & Cailleau, 1990). If a factor is secreted by Sertoli cells in order to influence Leydig cell function, it must travel through interstitial fluid (IF), and it has been shown that the addition of IF to cultured rat Leydig cells results in stimulation of testosterone production (Sharpe & Cooper, 1984).

Many studies have examined the effects of staged seminiferous tubule segments on Leydig cell function, either using co-culture or by preparing seminiferous tubule conditioned medium (STCM). Results from such studies have been highly variable. Co-culture of seminiferous tubules at any stage with Leydig cells has been reported to enhance both basal and hCG-stimulated testosterone production (Verhoeven & Cailleau, 1986, Papadopoulos *et al.*, 1987b), to have no effect on basal or hCG-stimulated testosterone production (Parvinen *et al.*, 1984), or to have no effect on basal testosterone production but to inhibit hCG-stimulated testosterone production (Syed *et al.*, 1988). To confuse things further, some authors have reported that only tubules at specific stages can affect testosterone production. For example stage IX-I tubules have been reported to have an inhibitory effect (Vihko & Huhtaniemi, 1989). In contrast, Syed *et al.* (1985) reported that stages VIII-XI and XIII-I stimulated basal and hCG-stimulated testosterone production. In one experiment, STCM prepared from human seminiferous tubules was found to stimulate basal and hCG-stimulated testosterone production by human Leydig cells (Verhoeven & Cailleau, 1987). One of the aims of this thesis was to clarify how the addition of conditioned medium or the presence of seminiferous tubules in the culture dish could affect Leydig cell testosterone production.

Attempts have been made to purify the Sertoli cell factor(s) that stimulates steroidogenesis. The active stimulatory principle in both human and rat STCM has an M_r of 10-30kDa (Verhoeven & Cailleau, 1986, 1987). It appears that seminiferous tubules may secrete several factors which affect Leydig cell function, as Syed *et al.* (1988) measured an

inhibitory activity in STSM with an M_r of 40-50kDa. Human Sertoli cells secrete a stimulatory factor with an M_r of 79kDa, which has been shown to be different from transferrin, albumin and testibumin (Papadopoulos, 1991). The stimulatory action of IF on steroidogenesis is thought to be mediated via a 57-75kDa glycoprotein (Jansz *et al.*, 1990).

There is no direct information on the nature of the communicators between Sertoli cells and Leydig cells, though there are several factors which have been shown to affect Leydig cell function *in vitro*, and for which there is some evidence of Sertoli cell production, for example, GnRH and AVP.

Sertoli cells *in vitro* produce a GnRH-like activity ('testicular GnRH'), which can also be detected in IF (Nagendranath *et al.*, 1983, Sharpe *et al.*, 1981). In the testis only Leydig cells have GnRH-receptors (Bourne *et al.*, 1980), and the addition of GnRH or its agonists to isolated Leydig cells has, in the short term, stimulatory effects on basal testosterone production both *in vivo* (Sharpe *et al.*, 1983) and *in vitro* (Sharpe & Cooper, 1982). However, the physiological role of testicular GnRH has not been established beyond doubt as the stimulatory effect of co-culture of seminiferous tubules on Leydig cell steroidogenesis was not affected by the presence of GnRH antagonists (Parvinen *et al.*, 1984). Attempts to purify testicular GnRH have shown that it is different from hypothalamic GnRH and that more than one form may exist (Bhasin *et al.*, 1983). To date testicular GnRH has not been isolated. There is some controversy about the direct role of GnRH in the human testis due to conflicting reports about the presence (Popkin *et al.*, 1985) and the absence (Clayton & Huhtaniemi, 1982) of GnRH receptors.

Another peptide which may have a role as a Sertoli cell-Leydig cell communicator is AVP. An AVP-like substance is present in rat testicular extracts (Kasson *et al.*, 1985) and in IF (Pomerantz *et al.*, 1988). AVP receptors have been localised to rat Leydig cells (Meidan & Hsueh, 1985) and addition of AVP to cultured rat Leydig cells has short-term stimulatory effects (Sharpe & Cooper, 1987). However, the physiological role of AVP in the testis is questioned by data showing an extremely low level of expression of AVP mRNA in the rat testis (Ivell, 1992).

Despite the knowledge that testosterone is vital for spermatogenesis, the means by which testosterone and other Leydig cell

products affect Sertoli cell function remain unknown. The only known effects of testosterone on Sertoli cells is to stimulate ABP and transferrin secretion, neither of which are directly involved in spermatogenesis (see Saez *et al.*,1987 for review). Recently, Sharpe *et al.* (1992) have used the EDS model to deplete rat testes of Leydig cells and used 2-dimensional SDS-PAGE to examine radiolabelled proteins secreted by Leydig cell-depleted rats either with or without exogenous testosterone. In this way seven androgen regulated proteins were identified. Using stage-dissected seminiferous tubules the study demonstrated that the effects of testosterone on protein secretion were restricted to the stage VI-VIII grouping.

2.4.3. Interactions between peritubular myoid cells and other testicular cells

Peritubular cells are found in the basement membrane of seminiferous tubules and consist of one or more layers of contractile myoid cells in association with undifferentiated mesenchymal cells. Sertoli cells and peritubular cells are often found in direct contact (Schulze, 1984). Sertoli cells and peritubular cells are thought to produce separate components of the basement membrane (Skinner *et al.*, 1985) and co-culture of Sertoli cells with peritubular cells increases the attachment and viability of Sertoli cells (Tung & Fritz, 1980). The presence of peritubular cells also stimulated the production of ABP by Sertoli cells and it was postulated that peritubular cells might influence Sertoli cell functions (Tung & Fritz, 1980). A factor designated P-Mod-S was purified from conditioned medium from peritubular cell cultures. P-Mod-S stimulates Sertoli cell protein production and secretion, including ABP and transferrin, which are widely used as markers of Sertoli cell function (Skinner *et al.*, 1988). P-Mod-S also stimulates the production of inhibin by Sertoli cells (Skinner *et al.*, 1989). Isolated peritubular cells respond to androgens, and the production of P-Mod-S is believed to be controlled by androgens (Skinner & Fritz, 1985). Thus, Leydig cells produce testosterone in response to LH, and testosterone stimulates the production of P-Mod-S which then modulates Sertoli cell functions (see Skinner, 1991 for review). P-Mod-S does not appear to be involved in bidirectional communication by also

mediating Sertoli cell effects on Leydig cells, as Risbridger & Skinner (1992) have shown that P-Mod-S and other peritubular cell proteins do not affect steroidogenesis by cultured Leydig cells.

2.4.4. Interactions between Sertoli cells and germ cells

The cyclicity of Sertoli cell morphology and function, in an environment where each Sertoli cell is believed to be exposed to equal levels of gonadotrophins, strongly implies that Sertoli cell function is modulated by germ cells. Sertoli cell nuclear morphology and cellular lipid content vary cyclically (see Sharpe, 1983 for review). As has been discussed previously, the testosterone requirement of Sertoli cells varies in a cyclical manner, as do the number of FSH receptors. The maximum expression of FSH receptors is found at stage I, and the minimum at stages VI-VIII. These changes are reflected by simultaneous changes in the amounts of cAMP produced in response to FSH (see Parvinen, 1992 for review). Other cyclical changes in Sertoli cell activity can be related to specific events of the spermatogenic cycle. Sertoli cells secrete a protease plasminogen activator (PA; Lacroix *et al.*, 1977) in a cyclical manner, with maximum secretion at stages VII-VIII (Vihko *et al.*, 1984). It is at these stages that spermiation and translocation of preleptotene spermatocytes to the adluminal compartment occur. Ingestion of residual bodies, spermiation and translocation all require restructuring and modification of Sertoli cell cytoplasm, and for these reasons the secretion of PA is thought to be maximal at these stages (Sharpe, 1986).

Direct evidence for Sertoli cell-germ cell interactions comes from studies examining the functions of Sertoli cells *in vitro* following experimental alteration of the germ cell population *in vivo*. Germ cells can be depleted from the testis by X-irradiation, cryptorchidism, local heating or by administration of the germ cell specific toxin, methoxyacetic acid (MAA). Further evidence has come from coculturing Sertoli cells with specific types of germ cells. For example the addition of enriched preparations of pachytene spermatocytes or round spermatids to cultures of immature rat Sertoli cells modulates the secretion of oestradiol (Jegou

et al., 1988), transferrin (Le Magueresse *et al.*, 1988) and ABP (Galdieri *et al.*, 1984).

Djakiew & Dym (1988) investigated the feedback between germ cells and Sertoli cells using a bicameral culture chamber which allows Sertoli cells to form confluent epithelial sheets between which Sertoli cell tight junctions form. Immature rat Sertoli cells were shown to secrete the majority of their proteins into the apical rather than the basal medium. Pachytene spermatocytes were isolated from adult rats and conditioned medium collected. The addition of pachytene spermatocyte conditioned medium to the apical surface of cultured Sertoli cells resulted in an increase in overall protein secretion by Sertoli cells. In addition at least two proteins were secreted by Sertoli cells in response to pachytene spermatocyte conditioned medium that were not observed in control Sertoli cell cultures. Thus it appears that pachytene spermatocytes are able to alter the composition of the surrounding milieu by modifying Sertoli cell protein secretion.

In a similar experiment, conditioned medium collected from round spermatids isolated from adult rats was lyophilised to prepare a concentrate of round spermatid proteins. The addition of round spermatid proteins to immature Sertoli cells cultured in bicameral chambers stimulated Sertoli cell protein secretion. Round spermatid proteins affected the polarity of Sertoli cell protein secretion, by stimulating apical more than basal secretion (Onoda & Djakiew, 1990).

Pachytene spermatocytes and round spermatids may affect Sertoli cell protein secretion by increasing gene expression. Coculturing immature Sertoli cells with either pachytene spermatocytes or round spermatids increases the expression of the PPenk gene, which is an opioid precursor gene found in Sertoli cells (Fujisawa *et al.*, 1992).

An important drawback of the *in vitro* approach is that isolated Sertoli cells are prepared from immature rats and are cocultured with germ cells prepared from adult rats. Immature Sertoli cells *in vivo* have only been exposed to pachytene spermatocytes and it may be that various functions of Sertoli cells are controlled by the most mature germ cells present in accordance with the progressive maturational changes in Sertoli cell function (Allenby *et al.*, 1991, Jegou, 1991, Sharpe, 1992). A technique has been developed to circumvent this problem; the glycol

ether methoxyacetic acid (MAA) is a germ cell specific toxin which depletes the testis of pachytene spermatocytes without inducing any other adverse effects (Creasy *et al.*, 1985). The occurrence of maturation depletion allows the generation of testes with successive but selected populations of germ cells missing.

This technique was used by Bartlett *et al.* (1988) to demonstrate that the absence of pachytene spermatocytes or elongate spermatids, but not of round spermatids, could increase the levels of serum FSH and ABP. These changes were found to be independent of changes in the levels of serum FSH and the levels of testosterone measured in serum or IF. Hence, *in vivo* studies also suggest that Sertoli cell function is modified by germ cells. More specifically, germ cell depletion *in vivo* using MAA has demonstrated that seminiferous tubule lumen diameter may be regulated by elongate spermatids. The diameter of the lumen and the area of the seminiferous epithelium vary cyclically. Lumen size is maximal at stages VII and VIII and minimal immediately after spermiation at stage IX (Wing & Christensen, 1982). In the absence of elongate spermatids the increase in lumen size normally seen at stage VII fails to occur. The absence of no other germ cell type had this effect (Sharpe, 1989b).

The volume of IF is believed to be controlled by Sertoli cells (Maddocks & Sharpe, 1989b). Maturation depletion of germ cells using MAA has shown that only the absence of late spermatids can affect the volume of IF. In the absence of elongate spermatids IF volume increased, again suggesting a role for germ cells in modifying Sertoli cell function. The absence of elongate spermatids is also closely associated with a marked decrease in the secretion of immunoactive Sertoli cell inhibin *in vitro* and decreased plasma levels *in vivo*, a change associated with increased levels of serum FSH (Allenby *et al.*, 1991).

Three potential routes by which late spermatids might influence Sertoli cell function are outlined by Jegou *et al.* (1992). Firstly, alterations in the type of adhesive contact between late spermatids and Sertoli cells may influence signalling between the two cells. Secondly, there may be a direct cytoskeletal link between the late spermatid-Sertoli cell interface and the nuclear transcription machinery so that externally imposed changes in shape might directly influence Sertoli cell gene transcription. Finally, when Sertoli cells phagocytes spermatid residual bodies they are

ingesting as much as 70% of the total spermatid cytoplasm. It has been proposed that residual bodies may regulate Sertoli cell function via mRNA coding for a factor(s) which controls Sertoli cell gene expression or more simply that phagocytosis triggers the production of a factor which regulates Sertoli cell and/or germ cell function.

As Sertoli cells support germ cells through all their developmental stages they obviously have a great influence over germ cell function. For example, spermatocytes and spermatids require lactate to support metabolism but are unable to convert glucose and thus depend on Sertoli cells to meet their energy requirements (Jutte *et al.*, 1982). In addition, Sertoli cells produce a mitogenic substance named seminiferous growth factor (SGF) which is believed to have a role in promoting the proliferation of spermatogonia (see Bellve & Feig, 1984 for review).

2.4.5. Germ cell-Sertoli cell-Leydig cell Interactions

In many situations when damage to the germ cell complement of the seminiferous epithelium affects Sertoli cell functions, changes in Leydig cell function are also apparent. As discussed above, Aoki & Fawcett (1978) demonstrated that damage to germ cells in the seminiferous epithelium was associated with Leydig cell hypertrophy. When germ cells are adversely affected by local heating, Sertoli cell function is disrupted secondary to the initial effect of the heat. The changes in Sertoli cell behaviour are associated with disrupted Leydig cell function and normal Leydig cell function is restored in concert with the recovery of the germinal epithelium (Jegou *et al.*, 1984).

Rat IF contains a factor which stimulates testosterone secretion by isolated Leydig cells. However, IF collected from germ cell-depleted cryptorchid testes has a higher stimulatory effect than control IF (Sharpe & Cooper, 1984). Similarly, Papadopoulos *et al.* (1987b) demonstrated that seminiferous tubule conditioned medium collected from irradiated rats (missing ~95% of their germ cells) had a higher stimulatory effect on isolated rat Leydig cells than medium collected from control cultures.

Studies such as these suggest a cellular interaction that extends from germ cells to Sertoli cells to Leydig cells, yet do not eliminate the possibility that changes in Leydig cell function are related simply to

disrupted Sertoli cell function. A direct effect of germ cells on Leydig cells via an effect on Sertoli cells has been demonstrated *in vitro*. Onoda *et al.* (1991) showed that immature rat Sertoli cells cultured in bicameral chambers secrete a factor which stimulates steroidogenesis in cultured rat Leydig cells and 80% of this bioactivity is secreted in a basal direction. The addition of pachytene spermatocyte proteins to the apical compartment of the bicameral chamber inhibited the basally directed Sertoli cell secretion of the Leydig cell stimulatory factor. Co-culture of pachytene spermatocytes and Sertoli cells resulted in a similar inhibition of production of the Leydig cell stimulatory factor. Addition of round spermatid proteins or co-culture of round spermatids and Sertoli cells did not inhibit the production of this factor to any great extent. Thus, the Sertoli cell secretion of a protein(s) which stimulates Leydig cell steroidogenesis is specifically modified by pachytene spermatocytes.

Total protein secretion by cultured rat seminiferous tubules has been assessed by 2D-SDS PAGE (Sharpe *et al.*, 1992). The production of seven proteins was found to be androgen-dependent. The influence of germ cells on Sertoli cell protein secretion has been examined using MAA to deplete specific germ cell types *in vivo* and looking for changes in the proteins secreted by seminiferous tubules *in vitro*. This technique has been used to demonstrate how germ cells can affect seminiferous tubule function, but surprisingly it was also found that the secretion of the androgen-dependent proteins was dependent on a normal complement of germ cells (McKinnell & Sharpe, 1992). Thus testicular function appears to be regulated by a complex, but highly organised cascade of cell-cell interactions which operates in both directions across the interstitium and seminiferous epithelium.

2.5. Aims of this thesis

This review has discussed the organisation and regulation of testicular function. In particular it has shown how the structured anatomy of the mammalian testis and the complexity of spermatogenesis suggest the need for local regulation, mediated by factors other than pituitary gonadotrophins. This review has tried to emphasise that the little known

about the regulation of testicular function has been determined in species other than man. Consequently there is a sparsity of information about human testicular function and many of the discoveries made in other species still need to be related to man.

The aims of this thesis were two-fold and centred on the use of human Leydig cells *in vitro* and the use of *ex vivo* techniques in the rat to clarify the cascade of regulatory interactions between germ cells, Sertoli cells and Leydig cells. To achieve these aims, a morphological examination of the relationships between Leydig cells and other cells in the human testis was made (chapter 4). The ability of human Leydig cells to function *in vitro* was determined (chapter 5), and the action on human Leydig cells, of factors believed to mediate communication between Sertoli cells and Leydig cells in the rat, was investigated in chapter 6. Toxicants known to affect the function of rat Leydig cells were tested for their ability to disrupt human Leydig cell function (chapter 10).

Cell interactions in the rat testis were assessed by depleting specific germ cell types *in vivo* and determining the effects on Leydig cell functions such as testosterone (chapter 7) and protein (chapter 8) production *in vitro*. Further assessment of these interactions was achieved by coculturing Leydig cells and seminiferous tubules or by adding seminiferous tubule conditioned medium to Leydig cells (chapter 9).

3. General Materials and Methods

This chapter describes techniques common to a number of studies in the thesis. Methods unique to specific experiments are described in the relevant chapters.

3.1. Tissue Supply

3.1.1. Animals

All animals used in these studies were male Wistar rats bred in the MRC Reproductive Biology Unit in Edinburgh. Rats were housed under a conventional 12h light : 12h dark cycle at 21°C with food and water available *ad libitum*. Animals were killed by asphyxiation with 100% CO_2 followed by cervical dislocation.

3.1.2. Patients

Testicular tissue was collected from 29 men, aged 54-89 years (mean ± S.D.= 72.9±9.5), who were undergoing orchidectomy as the primary treatment for prostatic carcinoma. No patient had any endocrine disease or any chronic systemic illness known to affect Leydig cell function.

3.2. Solutions for Tissue Dispersion and Cell Culture

3.2.1. Preparation of media

Incubation medium 1 (rat). Incubations of rat testes were in medium 199 containing Hank's salts and 20mM HEPES buffer (M199H; Flow Labs, Irvine, Scotland) supplemented with 0.5mg/ml collagenase (143 units/mg, Worthington Biochemical Corporation, Cambridge Bioscience, Cambridge) , 0.1% trypsin inhibitor (Sigma Chemical Co., Poole, Dorset) and 1.5mg/ml Bovine Serum Albumin (BSA; Fraction V, Sigma).

Incubation medium 2 (human). Incubations of human testes were in M199H supplemented with 0.75mg/ml collagenase, 0.1% trypsin inhibitor and 2.5mg/ml BSA.

Dispersion medium. Cells were washed and harvested in M199H containing 0.5mg/ml BSA.

Culture medium. Cells were incubated for periods of either 4 or 20 hours in medium 199 containing Earle's salts (M199E; Flow Labs, Irvine, Scotland), 0.5mg/ml BSA, 2mM L-glutamine (Gibco Ltd., Paisley, Scotland), 100 IU/ml penicillin (Gibco) and 100µg/ml streptomycin (Gibco).

3.2.2. Formation of discontinuous Percoll gradients

Percoll (Pharmacia Ltd. U.K.) contains colloidal silica coated with polyvinyl-pyrrolidone to reduce toxicity. It's low osmolality (20 mOsmoles/kg H_2O) means that before use it must be rendered isotonic. To achieve this, stock Percoll was mixed with 10 times normal strength M199H. This gives a solution, termed isotonic Percoll, with a density of 1.12 g/ml. This was then diluted further with M199H to form the constitutive layers of a discontinuous gradient. These were comprised of 5 distinct bands with densities of 1.0, 1.03, 1.05, 1.07, and 1.09 g/ml. The relative proportions of isotonic Percoll and M199H needed to form layers of these densities are shown in Table 3.1. Gradients were formed by layering the bands by hand using a 0.5x90mm spinal needle (Becton Dickinson, Oxford) and beginning with the 1.09mg/ml layer. Gradients were stored overnight at 4°C but were allowed to reach room temperature before use.

3.3. Preparation of Leydig cells

3.3.1. Preparation of rat Leydig cells

Leydig cells were isolated from the testes of 4 rats aged 70-120 days. Testes were removed via an incision in the scrotal sac, cut free from the

epididymides and during collection were kept in M199H pre-warmed to 37°C. The testes were then decapsulated and placed in pairs into 7mls incubation medium 1 in four 25ml universal containers (Mackay and Lynn, Edinburgh). The tissue was incubated in a shaking water bath at 70 cycles/min for 40-50 min at 37°C . The universals were arranged so that the shaking motion was longitudinal. The contents of each universal were diluted with dispersion medium, and the universal was swirled forty times. Tissue and tubule fragments were allowed to settle and the cell suspension was collected in a 25ml syringe and filtered through a 2.5 inch Swinnex filter (Millipore, Hertfordshire), containing 60μm gauze to remove undispersed tissue and tubule fragments. This step was repeated but with a gentler mixing action. The filtrate was centrifuged (Minifuge T, Heraeus Christ, Germany) at 160*g* for 10 min at room temperature, and the sedimented cells resuspended in a total of approximately 10ml of dispersion medium to give a crude interstitial cell suspension.

3.3.2. Percoll purification of rat Leydig cells

The crude cell suspension was loaded onto 2 Percoll gradients in 5ml batches and centrifuged at 500*g* for 24 min at room temperature. The band that contains Leydig cells is located between the layers with a density of 1.07 and 1.09g (Simpson *et al.* 1987), (see Figure.3.1.). The cells were aspirated using a plastic Pasteur pipette (Becton Dickinson, Oxford), washed in dispersion medium and harvested by centrifugation at 200*g* for 10 min at room temperature. Sedimented cells were resuspended in 10mls dispersion medium, counted with a Neuberg haemocytometer and diluted to 250,000 cells/ml in culture medium. Cells were plated at a density of 50,000 cells/well in a total volume of 200μl in 48-well tissue culture plates (Co-star, Nucleopore U.K. Ltd., Buckinghamshire). Plates were incubated at 32°C in a humidified atmosphere of 95% air/5% CO_2 for an initial period of 2 hours ('pre-incubation'), after which time the medium was removed and fresh medium and treatment solutions were added to a total volume of 300μl. Medium was aspirated using a pipette at 4 and at 20h and stored at -20°C until analysed.

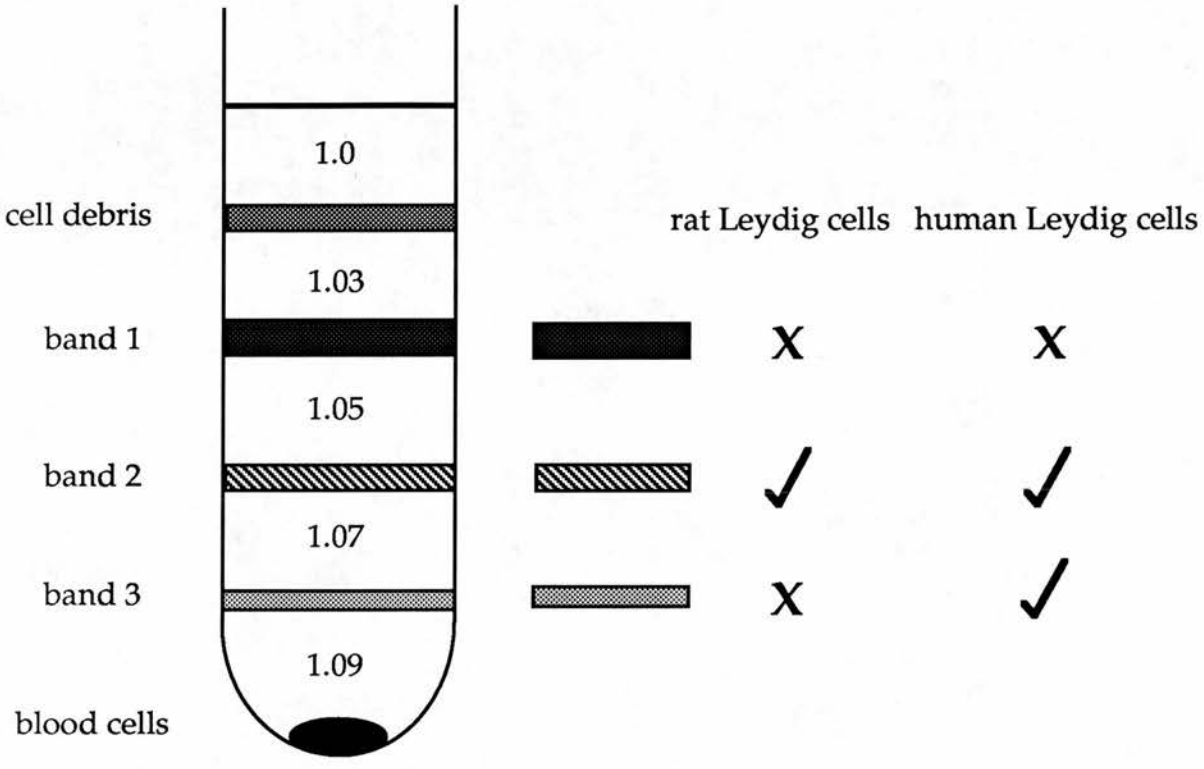
Table 3.1. Formation of discontinuous Percoll gradients.
Volumes of isotonic Percoll and M199H required for the preparation of fractions of increasing density used in the formation of discontinuous Percoll gradients.

Table 3.1. Formation of Discontinuous Percoll Gradients.

Density (g/ml)	Isotonic Percoll (ml)	Medium 199H (ml)
1.09	7.70	2.30
1.07	6.00	4.00
1.05	4.30	5.70
1.03	2.55	7.45
1.00	0.00	10.00

Figure 3.1. Schematic representation of the pattern of fractionation of rat and human testicular cells on discontinuous Percoll gradients .

Successive layers of Percoll with densities of 1.09, 1.07, 1.05, 1.03 and 1.00g/ml were used. The layers within which rat and human Leydig cells were concentrated are indicated on the right.


1.0
cell debris
rat Leydig cells
human Leydig cells
1.03
band 1
X
X
1.05
band 2
✓
✓
1.07
band 3
X
✓
1.09
blood cells

3.3.3. Preparation of human Leydig cells

Human Leydig cells were prepared for culture using adaptations of the method described above for the rat.
Testicular tissue was placed on ice immediately after excision and taken from the Western General Hospital to the Reproductive Biology Unit as quickly as possible (30-60 min after removal).
After weighing, the human testes were cut into approximately $10mm^3$ pieces and 8-10mgs of tissue placed in a 25ml universal containing 7mls of incubation medium 2. Dispersion occurred during an incubation for 45-60 min. Several blocks of tissue were processed for histological purposes and further blocks were processed so that the tissue would be suitable for immunocytochemistry (see below).
Cell suspensions were treated as above until the Leydig cells were harvested. Human Leydig cells concentrate in 2 layers on discontinuous Percoll gradients, namely at the 1.07/1.09g interface and at the 1.05/1.07g interface. (Simpson *et al.* , 1987), (see Figure. 3.1.). Both layers were aspirated and resuspended in a maximum volume of 5mls of culture medium. The cell plating regime was as described in section 3.3.2.

3.3.4. Assessment of cell viability

The viability of Leydig cell preparations was determined by measuring intracellular levels of adenosine triphosphate (ATP). This was done using the 1243-102 ATP monitoring kit from Bio-orbit, Berkshire. The assay is based on the quantitative measurement of light. Firefly luciferase is used to catalyse a reaction between ATP, luciferin and oxygen which results in the production of oxyluciferin, adenosine monophosphate and light. To measure ATP at the end of a culture period cells were washed with 0.1M Tris-acetate, 2mM EDTA buffer, pH 7.75 (assay buffer) and transferred from the bottom of the plates into fresh assay buffer using a rubber policeman. Concentrations of 50,000 cells in 100µl assay buffer were then sonicated for 30-60 seconds using an MSE Soniprep 150, (MSE instruments Ltd., U.K.). Samples were diluted in 200µl monitoring reagent and 600µl assay buffer, and this solution was diluted a further 1: 4 in assay buffer, before measurement of luminescence in a luminometer.

In order to calibrate the assay system a known concentration of ATP standard was then added to each sample and a second luminescence reading taken.
Typical ATP levels were between 200-600 pmoles/10^6 cells. No drug treatment used caused ATP levels to fall below 75% of the control value in that experiment.

3.3.5. Characterisation of Leydig cells

Leydig cells were identified histochemically by staining for 3ß-hydroxysteroid dehydrogenase (3ß-HSD). Aliquots of 250,000 cells/ml from each experiment were stored at -20°C until required. The staining solution consisted of 0.7mls 1.6mg/ml nicotinamide (Sigma) in 0.1M phosphate buffered saline (PBS), 0.8mls 3mg/ml NAD^+ free salt (Sigma) in PBS, 0.1mls 2mg/ml 5-androstan-3ß-ol-17-one (Sigma) in dimethyl formamide (BDH Ltd., Thornliebank, Glasgow), 1ml 1mg/ml nitroblue tetrazolium (NBT) (Sigma) in PBS and 4mls PBS. Defrosted aliquots of Leydig cells were centrifuged onto glass slides using a cytospin (Shandon-Elliott, U.K.). Samples were spun at 1000 rpm for 5 min. The slides were then incubated in NBT solution at 37°C for at least 30 min. If required, slides were fixed in 50% ethanol/10% formaldehyde for counting at a later date. Preparations were counted with a haemocytometer. A minimum of 5 fields of 100-150 cells were counted each time. Cells that were colourless were scored as negative and dark blue/purple cells were scored as Leydig cells. The percentage of Leydig cells in rat preparations ranged from 76-88%, and in human preparations from 62-77%.

3.4. Measurement of Testosterone

3.4.1. Iodination of testosterone tracer

Testosterone-3-carboxymethyl oximino-histamine (T-CMO) was iodinated by the Chloramine-T method (Hunter & Greenwood, 1962). The T-CMO conjugate was stored at -40°C as a 1µg/µl stock solution in ethanol. 5µg of the conjugate was dried down then redissolved in 40µl 0.5M phosphate buffered saline (PBS). 20µl Na ^{125}I (2mCi, Amersham

International, Aylesbury, Buckinghamshire) was added and the iodination initiated by the addition of 10µl fresh chloramine-T (1mg/ml in 0.05M PBS; BDH) solution . The mixture was vortexed and the reaction allowed to proceed for 2 min before being terminated by the addition of 10µl sodium metabisulphite solution (1mg/ml in 0.05M PBS; BDH).

3.4.2. Tracer purification

Unincorporated ^{125}I, labelled and unlabelled CMO-T were separated by HPLC (LKB Pharmacia Ltd., U.K.) using a 40-100% gradient of acetonitrile in 0.05% triflouroacetic acid (Aldrich, Poole, Dorset), at a pump speed of 0.4ml/minute. A Mini-Assay gamma counter (Type 6-20, Mini Instruments, Essex) was used to count the fractions. Fractions forming the peak of activity corresponding to ^{125}I-CMO-T (see Figure 3.2.) were pooled to obtain 100µCi/ml in ethanol. This was stored at 4°C for up to 2 months.

3.4.3. Testosterone standards

Standards were prepared from a solution containing 1mg testosterone in 100 ml ethanol. 500µl were diluted in 20ml ethanol to give a working stock solution. 640µl of this solution were evaporated to dryness under nitrogen vapour at 40°C and the residue reconstituted in 25ml PBS containing 10% gelatin (PGBS, pH 7.4, plus 0.01% thiomersalate).This gave a concentration of 640pg/100µl, which was the top standard. Standards of 320, 160, 80, 40, 20, 10, 5 and 2.5 pg/100µl were prepared by double dilution. All solutions were allowed to equilibrate at room temperature overnight before use. Standards were stored at 4°C for a maximum of 3 months. A representative testosterone standard curve is shown in Figure 3.3.

3.4.4. Assay procedure

Testosterone concentrations in Leydig cell incubation media were assayed in duplicate after appropriate dilution. 100µl aliquots of standards, samples and quality control media (high and low QCs) were incubated

with 500µl PGBS, 100µl testosterone antiserum (1: 350,000 dilution) and 100µl ^{125}I-CMO-T (approximately 12,000cpm).Two standard curves were included at the beginning and end of all assays. Standards and QCs were assayed in triplicate. Also included were tubes containing either 700µl PGBS (NSB) or 600µl PGBS plus 100µl testosterone antiserum (B_0). NSB tubes were used to measure non-specific binding of the tracer to the assay tubes, whilst the B_0 tubes determined the maximal binding of the tracer in the absence of testosterone. Tubes containing 100µl tracer only were used to determine the total amount of activity added to each tube (total counts; TC). After vortexing, the tubes were incubated at room temperature for 3 hours before the addition of 100µl normal sheep serum (1: 1000 dilution; Scottish Antibody Production Unit) and 100µl donkey anti-goat second antibody (1:25 dilution; Scottish Antibody Production Unit). After vortexing tubes were incubated at 4°C overnight.
Then, 1 ml wash buffer (0.9% saline plus 0.2% Triton X-100) was added to all tubes (except TCs). The assay was then centrifuged at 4°C for 30 min at 2500 rpm. The supernatant was removed and the tubes allowed to drain. The precipitate was counted for 60 seconds in a gamma counter (1261 Multigamma, LKB Wallac, Turku, Finland). Data was fed directly into an Apple Macintosh computer running the program Assayzap (Biosoft, Cambridge). Assay results (including fitted standard curves, % tracer binding and QC values) were generated directly by Assayzap.

3.4.5. Assay sensitivity and variation

The intra-assay coefficient of variation was 4.1%. The limit of sensitivity of the assay (defined as 2 standard deviations from the zero point) was 94% B/B_0 which was lower than the minimum standard used (i.e. <2.5 pg). The inter-assay coefficients of variation for the low (4.93 pg/tube) and high (81.15 pg/tube) QC's were 21% and 16.2% respectively.

Figure 3.2. Purification of ^{125}I-testosterone tracer.
HPLC elution profile of radioactivity.

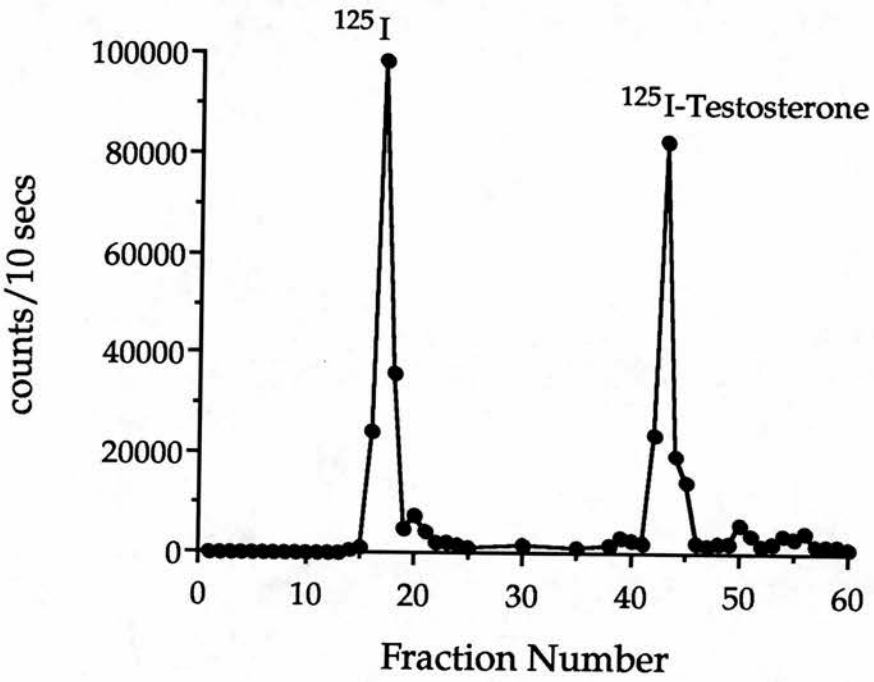
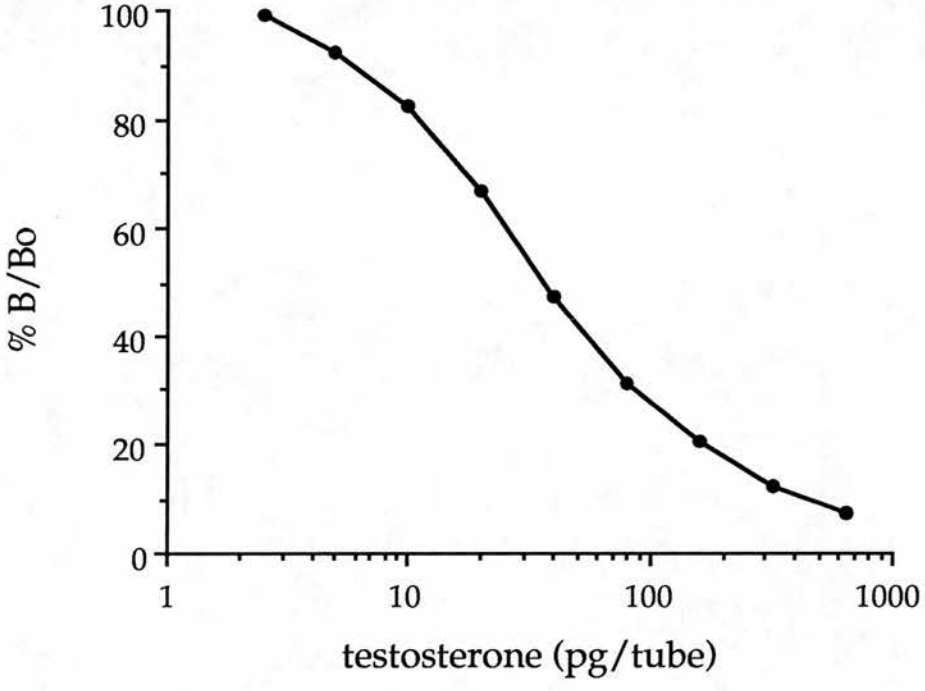

125I
125I-Testosterone
100000
80000
60000
40000
20000
0
counts/10 secs
0
10
20
30
40
50
60
Fraction Number

Figure 3.3. A representative testosterone standard curve.


100
80
60
40
20
0
% B/Bo
1
10
100
1000
testosterone (pg/tube)

3.5 Histology

All tissue fixation, processing, embedding, sectioning and toluidine blue staining was kindly performed by Mr Mike Millar.

3.5.1. Tissue fixation

Small blocks of tissue selected from at least three different areas of the testis were immersed in 'Superfix' solution (12% paraformaldehyde, 0.26% picric acid, 3% glutaraldehyde buffered with 0.2M cacodylate) overnight.

3.5.2. Processing of fixed testicular tissue

Tissue blocks were washed several times in 0.2M cacodylate buffer for a minimum of 4 hours, and then post-fixed in 2% osmium tetroxide (in 0.2M cacodylate) for 2-3 hours at 4°C. The cacodylate washes were repeated for at least 30 min before 2 further 15 minute washes in 0.05M maleate buffer. Blocks were then post-fixed in 1% uranyl acetate for 2 hours at room temperature, then washed again in maleate buffer for 2 periods of 15 min. After this the blocks were washed twice in increasing concentrations of ethanol (70,80,90 and 95%) for 10 min each, then several times in 100% ethanol for 1 hour. They were then washed twice in neat propylene oxide for 30 min, before being pre-embedded in a 50: 50 mixture (v/v) of propylene oxide: epon araldite for 24 hours. After this time the 50: 50 was poured off and the blocks placed in an oven at 60°C for 10 min to evaporate excess propylene oxide. Blocks were then transferred to liquid araldite for at least 6 hours and then into fresh liquid araldite for a minimum of 12 hours. The blocks were then embedded in fresh liquid araldite in flat-bottomed, cylindrical plastic capsules. These were placed in an oven at 60°C for 24-36 hours to allow the araldite to polymerize.

3.5.3. Sectioning

Following the removal of excess araldite from the edges of testicular blocks, semi-thin sections were cut to 0.5-0.75μM, using glass or diamond knives, on a Reichert Jung microtome, model no. 2050.
Paraffin blocks were sectioned to 3-4μM with a hand-operated microtome and a D-profile knife.

3.5.4. Staining

Plastic sections were stained by applying toluidine blue for a few seconds. The toluidine blue was always freshly filtered before use.

3.5.5. Microscopy and photography

Sections were examined and photographed using a Zeiss photomicroscope (Zeiss, Welwyn Garden City).

3.6. Data analysis

Experimental results were assessed initially by one-way analysis of variance (ANOVA) using a statistical package designed for the Apple Macintosh (CLR ANOVA; Clear Lake Research, Houston, Texas). Student's t-test was used for pairwise comparisons subsequent to ANOVA.

4. Characterisation of human Leydig cells

4.1. Introduction

Most studies of reproductive function are necessarily carried out in species other than man. The majority of studies concerning the function of Leydig cells have been performed in the rat or mouse. The use of one animal as a model for another is perfectly acceptable if sufficient data are available to validate the model. However this does not seem to be the case at present with respect to human Leydig cell function. The majority of studies performed with human testicular tissue have utilised whole testis pieces (Hsu *et al.*, 1978; Rodriguez-Rigau *et al.*, 1980; Huhtaniemi *et al.*, 1982; Bolton *et al.*, 1985), testicular homogenates (Hirsh *et al.*, 1981), or crude interstitial cell suspensions (Huhtaniemi *et al.*, 1982). Two of these studies (Huhtaniemi *et al.*, 1982; Bolton *et al.*, 1985) found that human Leydig cells were only weakly responsive to stimulation by hCG, showing a tenth of the response of rat Leydig cells to hCG. However the first study to use purified human Leydig cells (Simpson *et al.*, 1987) found that, allowing for individual variation, human Leydig cells were equally responsive to the effects of hCG as rat Leydig cells. Thus the finding of Huhtaniemi *et al.* (1982) that human Leydig cells were not regulated by hCG in the same way as rat Leydig cells can be attributed to the isolation and incubation techniques used. The experiments described in this chapter confirm in a large sample population (n=27) that, allowing for individual variation, highly purified human Leydig cells show a response to hCG that is comparable to the response shown by rat Leydig cells. They also prove that the population of Leydig cells isolated by Percoll gradient purification is analogous to the population present in the intact testis.

A major concern regarding the use of human testicular tissue is that the usual source is elderly men who are undergoing orchidectomy as treatment for prostatic carcinoma. It is feared that Leydig cells isolated from such a population would be unrepresentative of the younger population due to age-related changes in Leydig cell function (for review see Tsitsouras, 1987; Werner *et al.*, 1991) . While the exact influence of ageing on reproductive function remains unclear, the results of several

studies using testes from men of different ages have shown qualitatively normal spermatogenesis in the testes of elderly men. Sharpe *et al.* (1980) found that the number of LH receptors per Leydig cell did not decline with age, and Paniagua *et al.* (1987) demonstrated normal testicular histology up to the age of 80 in a significant proportion of a large elderly population studied. In fact many studies have been unable to correlate testicular function with age (Hsu *et al.*, 1978; Harman *et al.*, 1982; Johnson *et al.*, 1988). In the studies described in this chapter, using a population whose ages spanned 4 decades (54-89 years), no correlations were found between basic testicular functions and age.

Human Leydig cells exist as a heterogeneous population. At the light microscopic level Leydig cells are stained variably with toluidine blue, allowing a distinction to be made between light and darkly staining cells. Electron microscopy has shown that there is no difference in the quantity of membranes or in organelle composition between the two cell types (Schulze, 1984). An attempt was made to separate these two cell variants using the technique of elutriation, and to assess testosterone production in the populations distinguished in this way.

4.2. Experimental Procedures

4.2.1. Basic responsiveness of human Leydig cells

To establish basal and hCG-stimulated testosterone production, Leydig cells from every individual were incubated at a density of 50,000 cells/well in 200µl culture medium. After a pre-incubation period of 2h, this medium was removed and replaced with either 300µl fresh culture medium alone, or with 300µl culture medium containing a supramaximal concentration of hCG (30IU/ml; Chorulon, Intervet U.K. Ltd, Cambridge). A more detailed dose response to hCG (0.01-100mIU/ml) was undertaken on 4 separate occasions. In all cases, cells were incubated for a further 20h after addition of treatment, then medium was aspirated and stored at -20°C until assayed.

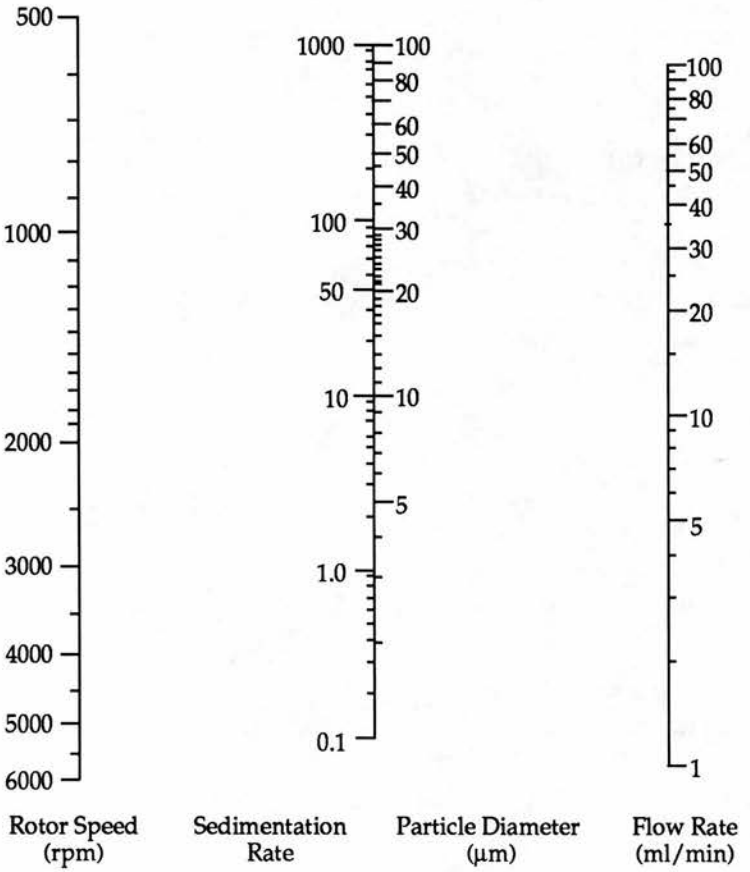
4.2.2. Comparison of the ratio of light: dark Leydig cells isolated by Percoll purification with the ratio found *in situ*

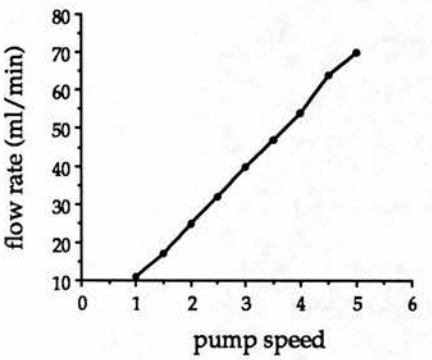
On two different occasions Leydig cells were Percoll-purified as described in chapter 3. The Leydig cell bands were sedimented as normal then resuspended in 2 ml dispersion medium. The number of cells was counted in a haemocytometer and sufficient cells removed to allow measurement of basal and hCG-stimulated testosterone production. The remaining cells were prepared for fixation as described below.
Cell counting was undertaken using a 100,000mm^2 graticule (Graticules Ltd., Tonbridge, Kent) in the eyepiece of a Zeiss photomicroscope. With sections prepared from isolated Leydig cell pellets the number of Leydig cells falling beneath the intersection points of the graticule were counted. This was done for 10 fields on a section from each Percoll layer. When counting Leydig cells in whole testis sections, the total number of Leydig cells in the area covered by the graticule was counted in 10 fields in two sections from different areas of the testis.

4.2.3. Elutriation of crude Leydig cell preparations

In an attempt to assess the extent to which human Leydig cells form a homogeneous population, and to try and separate light and dark Leydig cells, a crude Leydig cell suspension, prepared as described in chapter 3, was subdivided further using the technique of elutriation. A 10 ml suspension of crude Leydig cells in dispersion medium was subjected to elutriation (JE-6B elutriator rotor, Beckman Ltd.) at a constant rotor speed of 2000 rpm. Fractions were collected at different flow rates chosen to select out specific particle sizes. The elutriatior calibration curve and the flow rate nomogram are shown in Figure 4.1. Using the nomogram, flow rates were selected to allow separation of cells with average diameters of 14, 16 and 19μM. Cells concentrating in these 3 fractions were then spun at 160*g* for 10min to form a pellet. Each pellet was then resuspended in 2 ml dispersion medium and each layered onto a separate Percoll gradient. Leydig cell layers from each gradient (i.e. from each fraction) were collected as described in chapter 3, centrifuged at 200*g* then resuspended

Figure 4.1 Elutriation Calibration Curve and Flow Rate Nomogram


500
1000
2000
3000
4000
5000
6000
Rotor Speed
(rpm)
1000
100
50
10
1.0
0.1
Sedimentation
Rate
100
80
60
50
40
30
20
10
5
Particle Diameter
(μm)
100
80
60
50
40
30
20
10
5
1
Flow Rate
(ml/min)


flow rate (ml/min)
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
pump speed

in culture medium. The Leydig cell content of each fraction was determined using a haemocytometer, and sufficient cells removed to allow measurement of basal and hCG-stimulated testosterone production (triplicate wells). The remaining cell suspension was sedimented at 13,000 rpm for 10 min (Biofuge-A, Heraeus Sepatech) and the pellet then resuspended in 0.1M PBS. The centrifugation was then repeated, and the PBS removed and replaced with Superfix. A pellet from each fraction was then processed histologically, as described in Chapter 3. Medium from the dead volume on either side of the cell-containing fractions, was not taken through the Percoll purification step, but was sedimented at 160*g*, resuspended in 0.1M PBS and sedimented at 13,000 rpm for 10 min, as above, then resuspended in Superfix, and processed for histology.

4.3. Results

4.3.1. Basic responsiveness of human Leydig cells

The variability in basal testosterone production by isolated human Leydig cells is illustrated in Figure 4.2. Samples have been classified into 3 groups (low (n=9), medium (n=11) and high (n=7)) according to their ability to produce testosterone. A complete list of basal and hCG-stimulated testosterone production from each individual, together with details such as age, paired testicular weight and cell yield is given in appendix 1. Figure 4.2. shows that the medium and high testosterone producers each produced significantly more (p <0.01) testosterone than did the low producers. The difference in testosterone production between the medium and high group was also significant (p <0.05). The response to a supramaximal dose of hCG by Leydig cells within these 3 groups is illustrated in Figure 4.3. In all the groups hCG had a significant (p <0.01) stimulatory effect on testosterone production. Figure 4.4. shows the average fold response to hCG within each group. Although there was a suggestion that Leydig cells which produced more testosterone basally were more responsive to the effects of hCG than were Leydig cells with lower basal levels of testosterone production, analysis of variance showed no overall difference in responsiveness to hCG among the 3 groups (ANOVA; p =0.28).

In order to consider the responsiveness of human Leydig cells to hCG in more detail, 4 experiments were performed which determined the dose response of human Leydig cells to hCG (0.01-100mIU). Two experiments were with Leydig cells falling into the category of low testosterone production, and two experiments were with Leydig cells from the medium and high categories. Dose responses to hCG are shown in Figure 4.5. For both low and medium testosterone producers there was no significant effect of hCG below 10mIU. The effect of concentrations of 10 and 100mIU were significantly different from those seen at all other concentrations (p <0.01), but were not significantly different from each other. In the high production group significant stimulation of testosterone production (p <0.01) was seen at concentrations above 1mIU hCG, that is testosterone production by Leydig cells in this group was significantly stimulated by a lower concentration of hCG than was the case in the low and medium groups.

The possibility of a relationship between donor age or the general condition of the tissue samples, and the responsiveness of Leydig cells subsequently isolated from the tissue was considered. In order to test this hypothesis, factors such as age and testicular weight, which might have had some influence on *in vitro* responsiveness, were compared with experimental results such as basal or hCG-stimulated testosterone production, the fold response to hCG or total cell yield. Any statistical correlation between these factors was then determined (simple regression analysis, using the Statworks (Cricket Software, Ltd., London) package for the Apple Macintosh computer). The results are summarised in Figure 4.6. The correlation coefficient (r) between cell yield at the end of the isolation procedure and paired testicular weight (A) was 0.28, which was not significant. Basal testosterone production did not correlate significantly with age (C; correlation coefficient = 0.049) nor was there a significant correlation between the fold response to hCG and age (D; correlation coefficient = 0.028). The relationship between basal and hCG-stimulated testosterone production (B) was found to be statistically significant (correlation coefficient = 0.82, p <0.001,).

The possibility of a relationship between the condition of the testes and the responsiveness of Leydig cells subsequently isolated is considered briefly in Figure 4.7. Examples of the diversity in testicular morphology

found in samples from each of these groups are shown. There were no consistent differences in the condition of either the interstitium or of the seminiferous tubules between the low, medium and high *in vitro* testosterone producing groups. The relationship between testicular morphology and Leydig cell function *in vitro* is examined in more detail in chapter 5.

4.3.2. Comparison of the ratio of light: dark Leydig cells isolated by Percoll purification with the ratio found *in situ*

The ratio of light: dark Leydig cells found in layers of Leydig cells isolated on Percoll gradients was shown to be consistent with the ratio measured in sections of whole testicular tissue (Table 4.1.). It was not expected that the light : dark ratios would be so similar in different individuals (here nominated JL and JV), however the results discussed in chapter 5, confirm that the ratio of light : dark Leydig cells in the human testis is not constant between individuals. The morphology of each testis is shown in Figure 4.8., as is the composition of the cellular suspension isolated by Percoll-purification. The testicular morphology of JL and JV was comparable as was the *in vitro* testosterone production by Leydig cells isolated from these testes.

4.3.3. Elutriation of crude Leydig cell preparations

A summary of all details collected regarding elutriated fractions 2,3 and 4 is shown in Table 4.2. The majority of cells ($3.8x10^6$) were localised in fraction 2, which contained cells with an average diameter of 14μM. The types of cell located in each fraction were determined morphologically. Figure 4.9 consists of photographs showing the composition of each fraction. Light and dark Leydig cells are present in all fractions, and there was no significant difference between the ratios of light: dark Leydig cells in each fraction. In fractions 2, 3 and 4 this ratio was 1: 1.92, 1: 2.2 and 1: 1.6 respectively. Basal testosterone production by cells from fraction 2 was 0.8 ± 0.15 ng/10^6 cells, in fraction 3, 6.4ng/10^6 cells and in fraction 4, 46.86 ± 11.08 ng/10^6 cells. Figure 4.10 shows basal and hCG-stimulated

Figure 4.2. Basal testosterone production by human Leydig cell cultures.

A. Low testosterone producers (n=11). **B.** Medium testosterone producers (n=9). **C.** High testosterone producers (n=7). Each graph shows testosterone production over a 20h culture period. Each point is the mean±S.D. of n experiments. *p <0.05 significantly different from low group, **p <0.01 significantly different from low group, +p <0.01 significantly different from medium group.

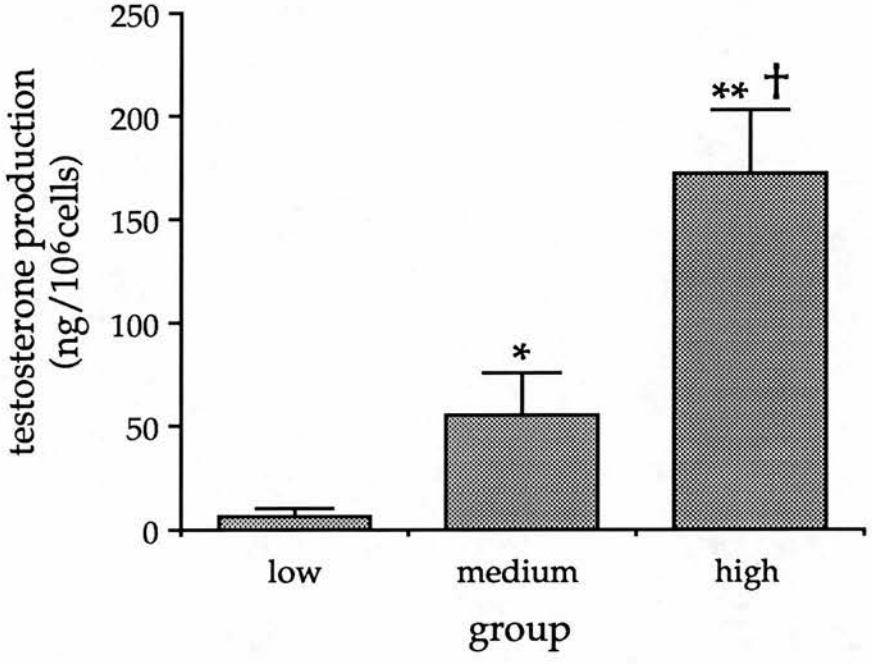

250
200
150
100
50
0
testosterone production
(ng/10^6cells)
*
** †
low
medium
high
group

Figure 4.3. The effects of 30IU/ml hCG on testosterone production by human Leydig cell cultures.

A. Low testosterone producers (n=11). **B.** Medium testosterone producers (n=9). **C.** High testosterone producers (n=7). Each graph shows testosterone production over a 20h culture period. The basal testosterone values shown in Figure 4.2. are repeated in these graphs for comparison. Each point is the mean±S.D. of n experiments. Note the differences in the scale of the y-axis between the groups. $^{**}p < 0.01$, significantly different from basal values.

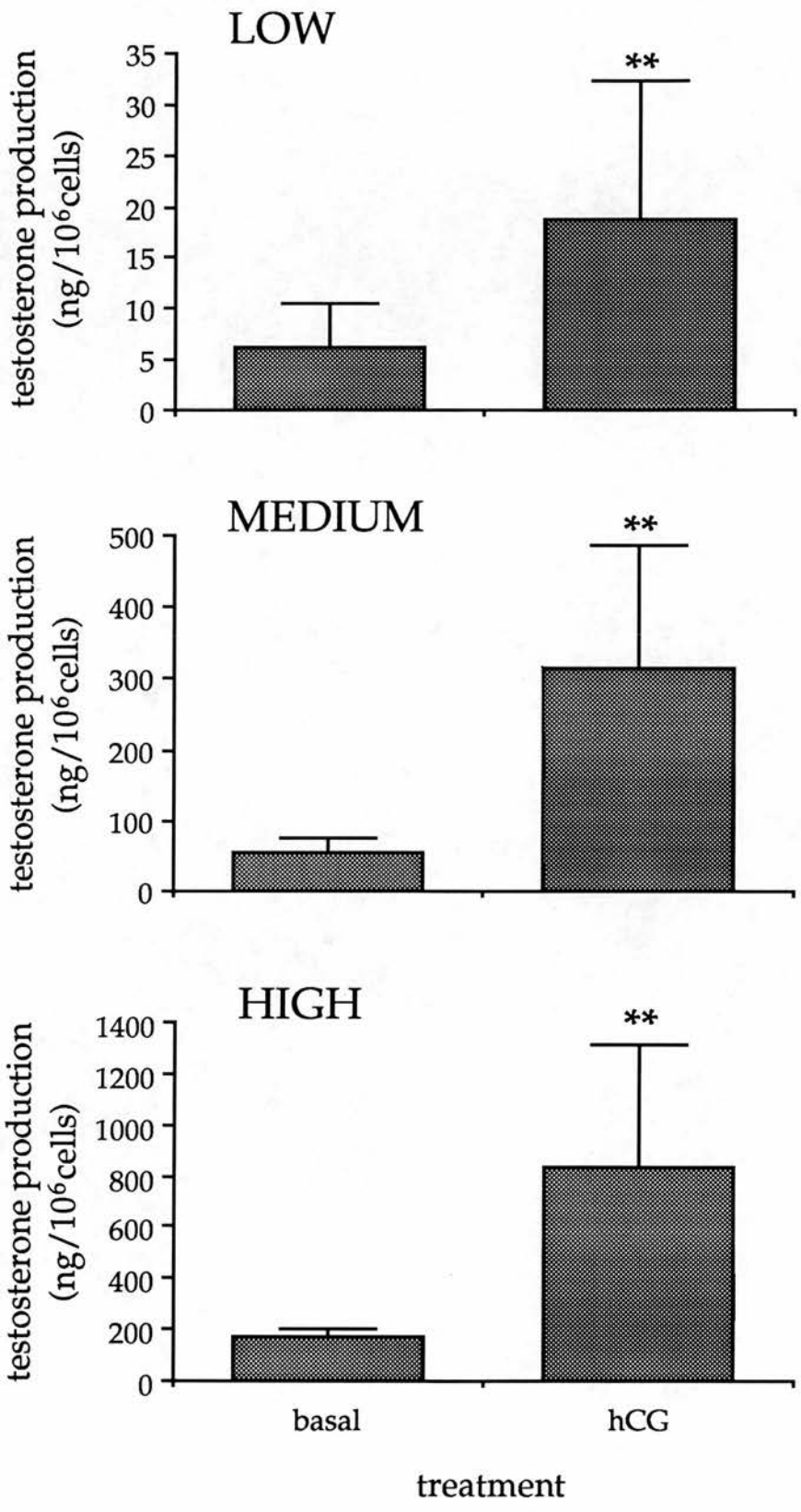

LOW
testosterone production (ng/10⁶cells)
35
30
25
20
15
10
5
0
**
MEDIUM
testosterone production (ng/10⁶cells)
500
400
300
200
100
0
**
HIGH
testosterone production (ng/10⁶cells)
1400
1200
1000
800
600
400
200
0
**
basal
hCG
treatment

Figure 4.6. Correlation curves of testicular parameters.
A. Correlation between cell yield *vs.* paired testicular weight, r = 0.28. B. Correlation between basal *vs.* hCG-stimulated testosterone production, r = 0.82, p <0.0001. C. Correlation between basal testosterone production *vs.* age, r = 0.049. D. Correlation between the fold response to hCG *vs.* age, r = 0.028.

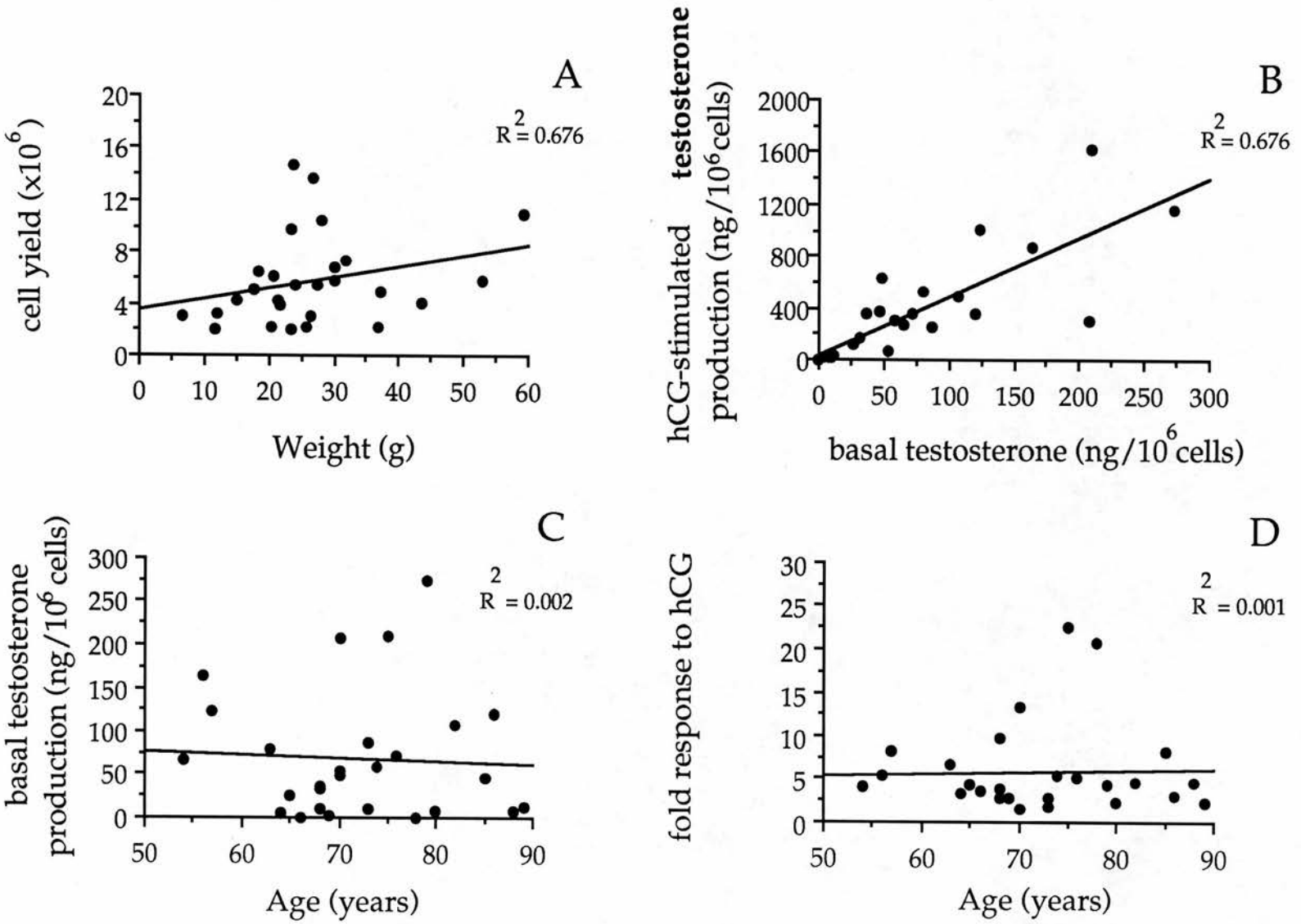

A
cell yield (x10^6)
Weight (g)
R^2 = 0.676
B
hCG-stimulated testosterone production (ng/10^6cells)
basal testosterone (ng/10^6cells)
R^2 = 0.676
C
basal testosterone production (ng/10^6 cells)
Age (years)
R^2 = 0.002
D
fold response to hCG
Age (years)
R^2 = 0.001

Figure 4.4. Magnitude of the response to hCG shown by human Leydig cell cultures.

A. Low testosterone producers (n=11). **B.** Medium testosterone producers (n=9). **C.** High testosterone producers (n=7). Each graph shows the fold response to 30IU/ml hCG over a 20h culture period. Each point is the mean±S.D. of n experiments. No significant differences were found (ANOVA, $p = 0.28$).

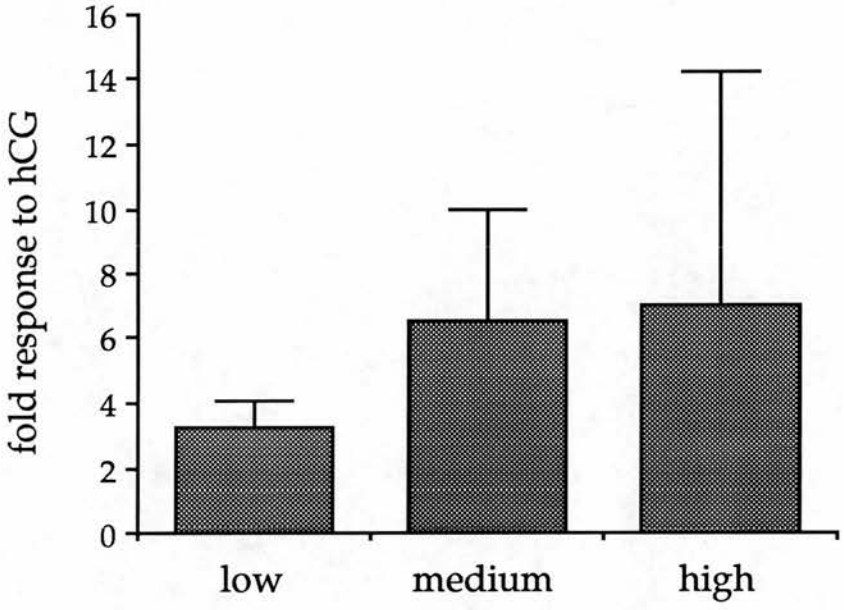

fold response to hCG
16
14
12
10
8
6
4
2
0
low
medium
high

Figure 4.5. The effects of increasing doses of hCG on testosterone production by human Leydig cell cultures.

A. Examples of hCG dose response in 2 low testosterone producers. **B.** A medium testosterone producer. **C.** A high testosterone producer. Each graph shows the response to 0.001-100mIU hCG over a 20h culture period. Each point is the mean±S.D. of triplicate wells in a single experiment. Note the differences in the scale of the y-axis between the groups. $*p < 0.05$,$**p < 0.01$, significantly different from basal values.

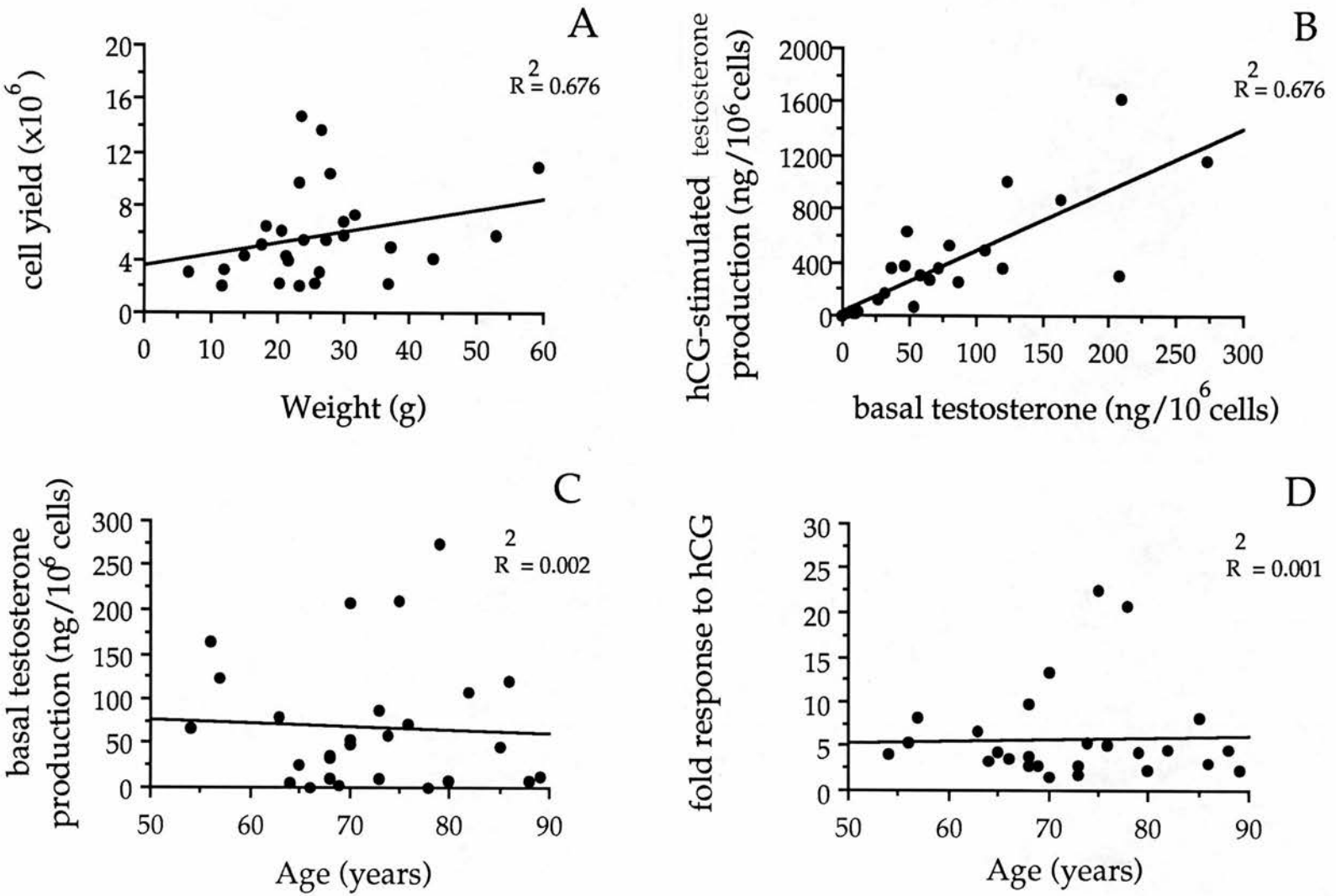
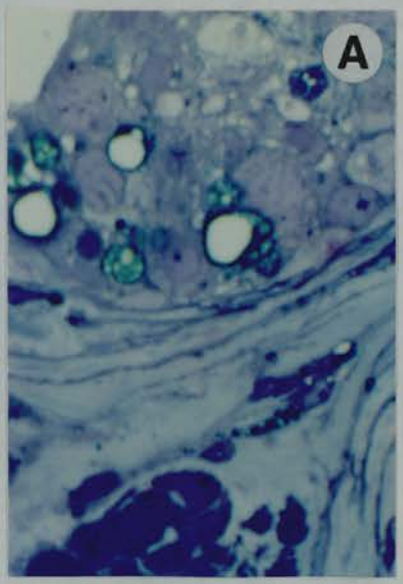
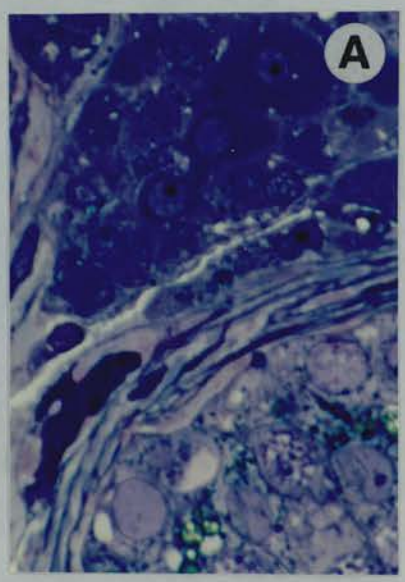
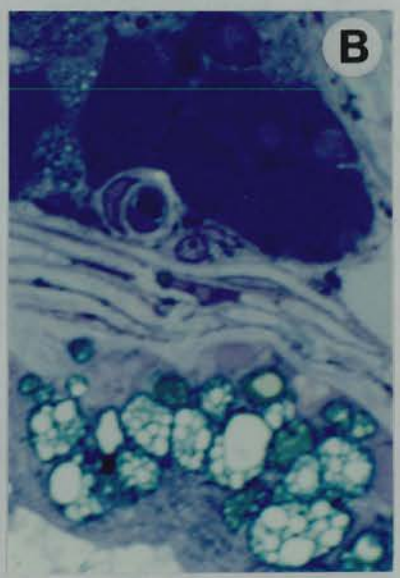

A
cell yield (x10^6)
R^2 = 0.676
Weight (g)
B
hCG-stimulated testosterone production (ng/10^6cells)
R^2 = 0.676
basal testosterone (ng/10^6cells)
C
basal testosterone production (ng/10^6 cells)
R^2 = 0.002
Age (years)
D
fold response to hCG
R^2 = 0.001
Age (years)

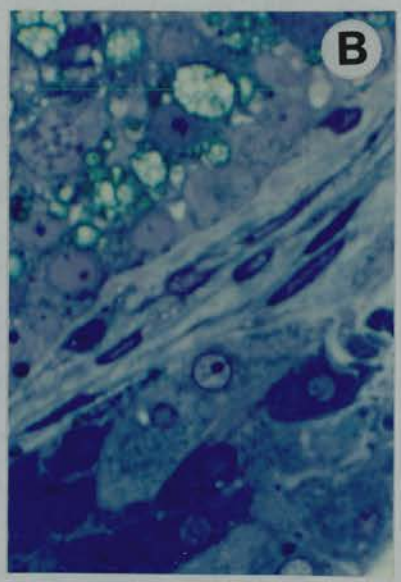
Figure 4.7 Histology of the human testis.

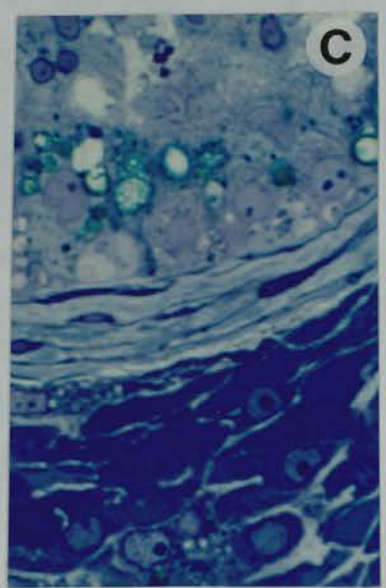
Examples of testicular morphology in : **A.** 2 low testosterone producers. **B.** 2 medium testosterone producers. **C.** 2 high testosterone producers.


A


A


B


B


C

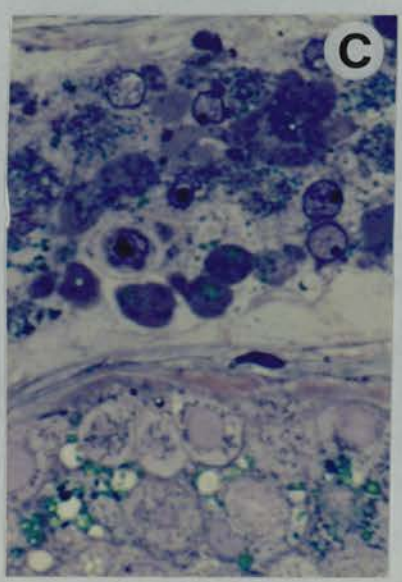
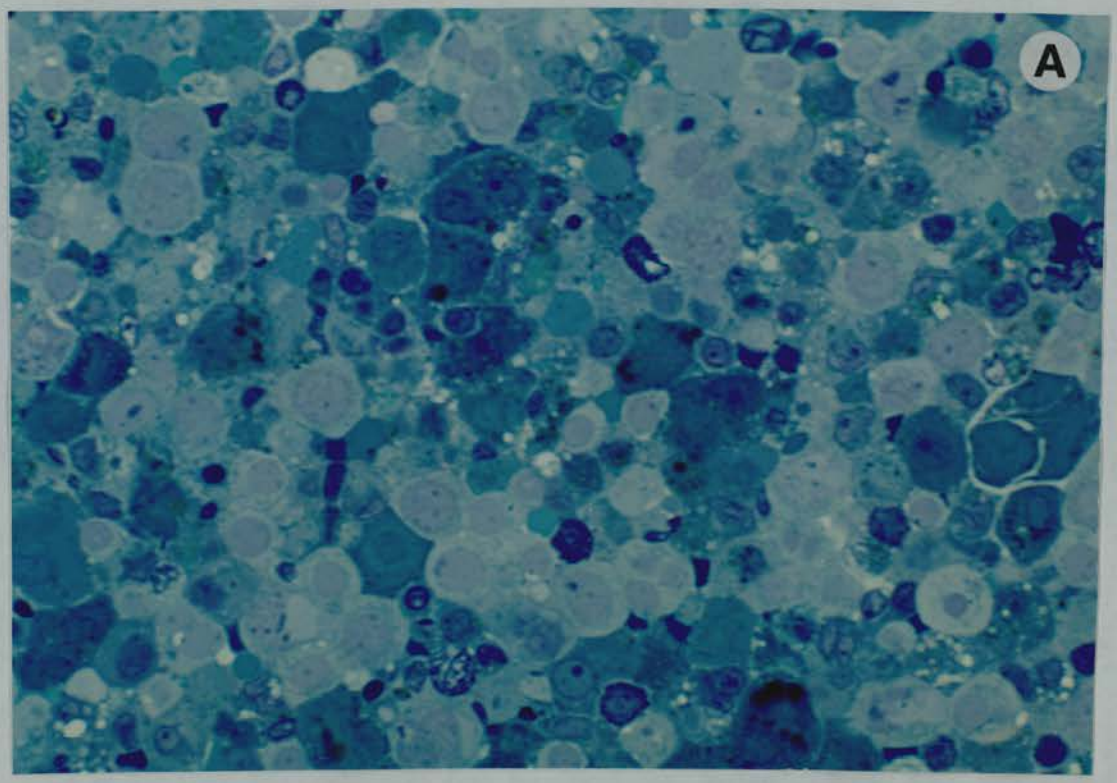

C

Figure 4.8 Cellular composition of human Leydig cell layers isolated on Percoll gradients.
A. Cells isolated from JV. **B.** Cells isolated from JL.


A

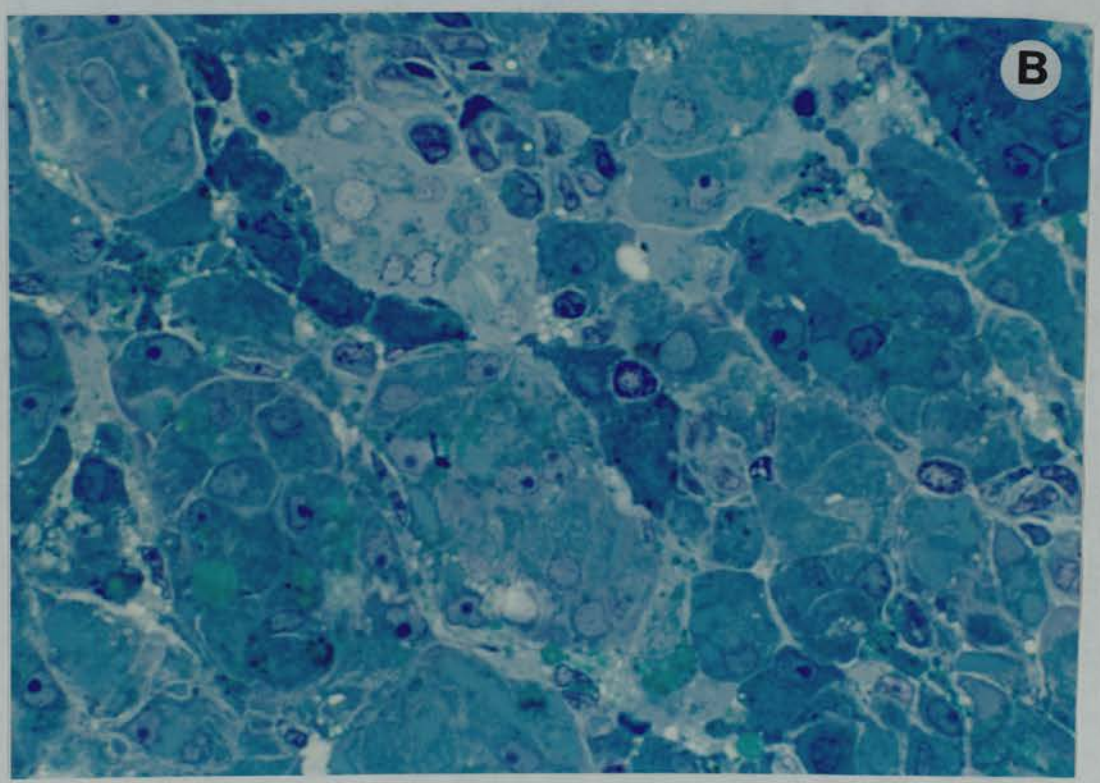
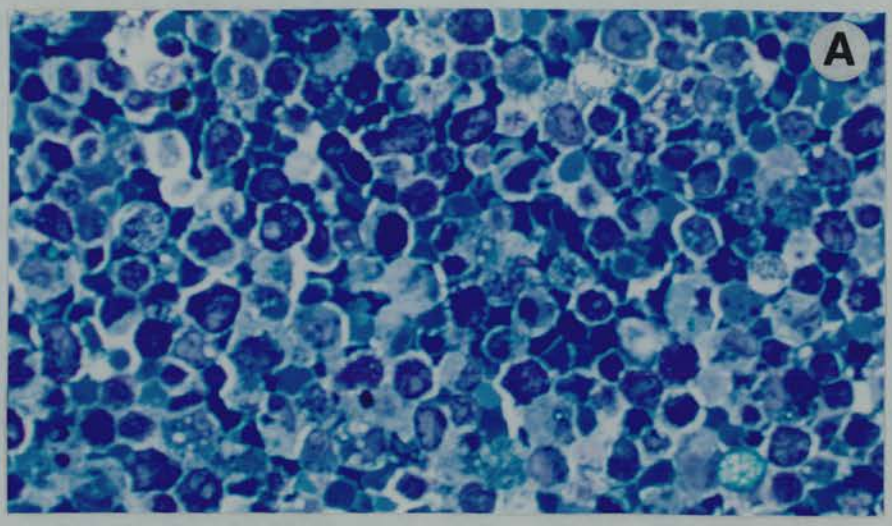

B

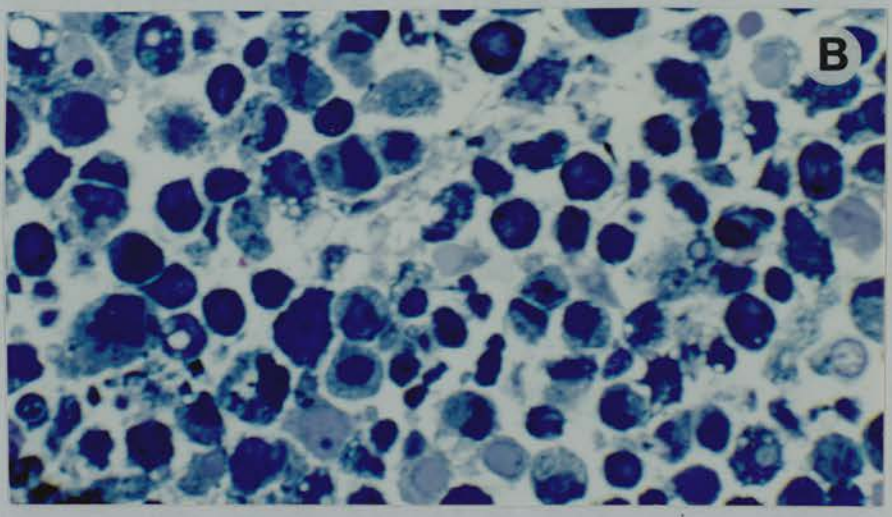
Figure 4.9 Cellular composition of human Leydig cell fractions separated by elutriation.

A. Fraction 2.

B. Fraction 3.

C. Fraction 4.


A


B

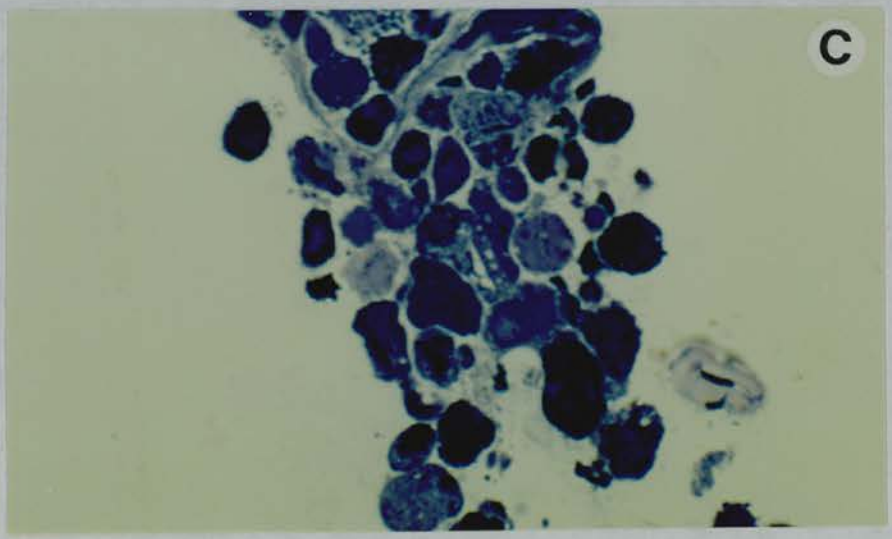

C

Figure 4.10 Basal and hCG-stimulated testosterone production by elutriated human Leydig cell fractions.

The graph shows basal testosterone production (b) and hCG-stimulated testosterone production (s) over a 20h culture period. Each column is the mean±S.D. of triplicate wells in a single experiment. Data without common superscripts are significantly different (a,b = p <0.01, c = p <0.05)

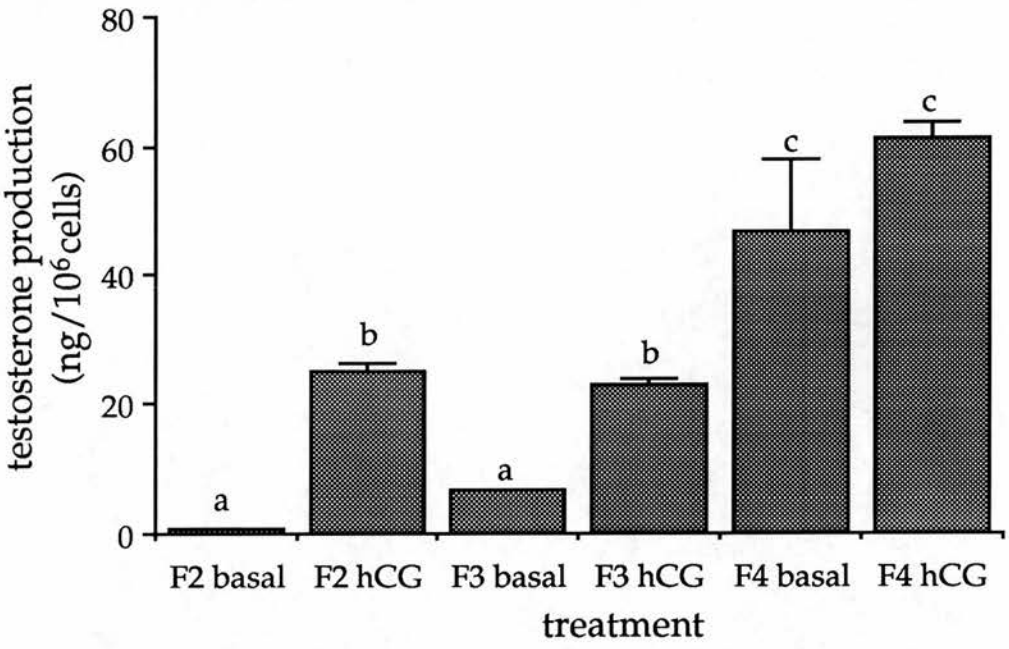

testosterone production
(ng/10^6cells)
80
60
40
20
0
a
b
a
b
c
c
F2 basal
F2 hCG
F3 basal
F3 hCG
F4 basal
F4 hCG
treatment

Table 4.1. Comparison of the numbers of light and dark Leydig cell numbers in the whole testis and in isolated Leydig cell suspensions.

Experimental and sample details from two individual men. Each value is the mean±S.D. of 20 fields for whole tissue sections, and of 10 fields for isolated Leydig cell suspensions.

Subject	JL	JV
Age (y)	84	82
testicular weight (g)	9.7	20.4
basal testosterone production ($ng/10^6$cells)	278.9	298.8
hCG-stimulated testosterone production ($ng/10^6$cells)	836.8	1596.1
no. light Leydig cells/ unit area (pellet)	54.25±5.7	52.63±5.85
no. light Leydig cells/ unit area (testis)	8.56±6.68	9.5±9.6
no. dark Leydig cells/ unit area (pellet)	66.75±5.65	68.13±5.44
no. dark Leydig cells/ unit area (testis)	10.56±5.95	13.8±8.2
ratio of light : dark Leydig cells (pellet)	1 : 1.23	1 : 1.29
ratio of light : dark Leydig cells (testis)	1 : 1.23	1 : 1.45

Table 4.2. Basic characteristics of human Leydig cells separated by elutriation.

Experimental and sample details.

fraction no.	cell diameter (μm)	cell yield ($x10^6$)	testosterone production ($ng/10^6$Leydig cells)
2	14	3.8	basal 0.8±0.15 hCG 24.98±1.38
3	16	0.795	basal 6.4±0.29 hCG 22.77±1.2
4	19	0.38	basal 46.86±11.08 hCG 61.14±2.57

testosterone production by Leydig cells in each fraction. Basal testosterone production was not significantly different between Leydig cells isolated in fractions 2 and 3, but basal testosterone production by Leydig cells in fraction 4 was significantly different ($p < 0.01$) from Leydig cells in both fractions 2 and 3. In fractions 2 and 3 hCG-stimulated testosterone production was significantly different ($p < 0.01$) than basal levels in both fractions, but were not significantly different from each other. Both basal and hCG-stimulated testosterone production by Leydig cells in fractions 2 and 3 were significantly different ($p < 0.01$) from basal and hCG-stimulated testosterone production by Leydig cells in fraction 4, although these were not significantly different from each other.

4.4. Discussion

The aims of the studies described in this chapter were threefold. Firstly, to investigate the ability of Leydig cells isolated from the testes of aged men to produce testosterone *in vitro*, both basally and in response to hCG. Secondly, to establish that the population of Leydig cells isolated by Percoll gradient purification is representative of the Leydig cell population existing *in situ*. This was achieved by comparing the ratio of light: dark Leydig cells found at the end of the isolation procedure with the ratio seen *in situ*. The final aim of the study was to separate light and dark Leydig cells and to compare their capability to produce testosterone *in vitro*.

Amongst the Leydig cells isolated from a total of 27 testes there was a marked variation in testosterone production measured *in vitro* and also in the response of the Leydig cells to hCG. Similar individual variations have been reported in studies which measured intratesticular testosterone content in whole testicular tissue fragments (Hirsh *et al.*, 1981; de la Torre *et al.*, 1982), in total testicular homogenates (Hsu *et al.*,1978), and in isolated human Leydig cells (Simpson *et al.*, 1987). The studies by de la Torre and Hirsh were carried out on testicular biopsy samples obtained from both fertile and infertile populations. These were shown to have comparable intratesticular levels of testosterone and to exhibit similar variability in testosterone production between individuals within the group. This is important as it demonstrates that variation in

intratesticular testosterone content occurs in a normal, young (26-48 years) population and is not particularly associated with Leydig cells isolated from an 'abnormal' population such as infertile men or, as in this study, elderly men. Although several studies have investigated the effects of ageing on reproductive function (for review see Werner *et al.*, 1991), it is still unclear how the functions of testicular cells decline as part of the ageing process. Most investigators have found elevated levels of serum FSH and LH in ageing men (Harman *et al.*, 1982; Deslypere & Vermeulen, 1984; Neaves *et al.*, 1984). The changes in serum testosterone levels, however, are much less clear. MacNaughton *et al.* (1991) and Deslypere & Vermeulen (1984) found that plasma testosterone concentrations decreased with age, while the study by Harman *et al.* (1982) found no effect of ageing on plasma testosterone levels. The relationship between age and serum gonadotrophin and testosterone levels was examined in more detail by Paniagua *et al.* (1987). In this study endocrinological and histological parameters were measured in 64 elderly men (51-90 years) undergoing orchidectomy. These data was compared with results obtained from 25 men (25-39 years) killed in traffic accidents. Paniagua *et al.* (1987) divided the elderly men into groups according to age and then subdivided each group into men whose testes showed complete spermatogenesis and normal or nearly normal histology (group A) and men whose testes showed maturation arrest of spermatogenesis in most tubules (group B), these distinctions being made after microscopic examination of fixed testicular sections. Up to 80 years of age, men in group A showed hormone levels and testicular parameters similar to those in the young control men. The implication for work on isolated human Leydig cells is that if histological examination reveals complete spermatogenesis together with relatively normal testicular morphology (as is the case for the majority of samples used in this study) this is indicative of a relatively normal population of testicular cells. Indeed, Simpson *et al.* (1987) found that men with poorly responsive Leydig cells *in vitro* could not be distinguished from those whose Leydig cells made large amounts of testosterone *in vitro* on the basis of plasma FSH, LH or testosterone levels. However, degenerative changes in testicular morphology do occur as part of the ageing process. One study which compared testicular tissue taken at autopsy from 2 groups aged 20-48 years

and 50-76 years, found that the mean total number of Leydig cell nuclei had decreased by 44% in the older age group, however the volume of Leydig cell nuclei was constant between groups (Neaves *et al.*, 1984). Seminiferous tubular boundary tissue is subject to age-related thickening (Johnson *et al.*, 1988), possibly making cell-cell communication more problematical. While these structural changes and also the increase in serum gonadotrophins suggest that Leydig cells isolated from aged human testes are not representative of the Leydig cell population in younger men, the results of one of the studies on large numbers of men (Paniagua *et al.*, 1987) found marked individual variations in all age groups. Neaves *et al.* (1984) also concluded that the response of the human testis to ageing is highly variable. Thus considering that the great majority of testicular tissue used in the studies described in this thesis showed complete spermatogenesis and relatively normal histology (see chapter 5) it can be assumed that despite their age they were representative of a total population that is itself highly variable in nature.

In this study Leydig cells were subdivided on the basis of their ability to produce testosterone *in vitro* into low, medium and high producers. There was no significant difference in the average ages of the men in these groups (75±9.3, 69.6±7.9, 72.1±11.8 years, respectively) nor did they deviate from the overall average age of 72.9±9.5. The amounts of testosterone produced by these groups differed significantly ($p < 0.01$ low *vs* medium or high, $p < 0.05$ medium *vs* high). The response of all groups to a supramaximal dose of hCG was significant ($p < 0.01$), and although there was a trend towards the Leydig cells which had a higher basal level of testosterone production being more responsive to the effects of hCG, this was not proven statistically. A study by Neaves *et al.* (1984) found that although there was a 44% reduction in the number of Leydig cells between 2 groups with age ranges of 20-48 years and 50-76 years, there was no change in Leydig cell nuclear volume. They suggested that the poor response of older men to hCG administration (Harman & Tsitsouras, 1980) was because the surviving Leydig cells are working close to their maximum capacity to compensate for their reduced numbers. However this study does not support the finding that Leydig cells in elderly men are unresponsive to hCG. Appendix 1 shows the response of all

preparations of isolated Leydig cells to a supramaximal dose of hCG. The average fold response was x6, with a range between x1.8 and x22.5. In fact the minimum response to hCG of x1.8, or 80% is equivalent to the average response to hCG of 91% in crude interstitial cell suspensions, and greater than the 41% response of whole tissue preparations reported by Huhtaniemi *et al.* (1982). Bolton *et al.* (1985) who performed hCG dose response tests in whole testicular pieces were unable to get greater than a 3-fold increase in testosterone production. They found that the maximum responses to hCG were seen at concentrations above 1IU hCG/ml. Figure 4.5 shows that with highly purified isolated Leydig cells, the low and medium testosterone producing group are significantly stimulated by a 100-fold lower dose of hCG (0.01IU), while the high testosterone producers are more sensitive to the effects of hCG by a further factor of 10 (0.0001 IU hCG). Thus the lack of response of isolated human testicular pieces or crude interstitial cell preparations that has been reported previously does not occur in the highly purified Leydig cells used in this study, suggesting that the lack of response described previously was a consequence of the techniques used in the isolation of Leydig cells. The ability of these cells to respond to hCG gives further support to the idea that the Leydig cells isolated by the technique described in this thesis are functioning normally.

An initial attempt at evaluating the interdependency of several testicular factors was unsuccessful. Cell yield at the end of the isolation procedure did not correlate with testicular weight, which reflects the individual variations in testicular morphology discussed earlier (Johnson *et al.*, 1988). Neither did cell yield correlate with age which at first seems to contrast with the study by Neaves *et al.* (1984) who found a decline in Leydig cell number with age. However, as most isolation techniques used to separate testicular cell types are able to isolate only a few percent of the total cell population (Simpson *et al.*, 1987), it is not too surprising that this *in vivo* change is not reflected in cell yields. Age was shown not to be a significant factor in determining either basal testosterone production *in vitro* or the fold response of the cells to hCG. A significant correlation ($p < 0.001$) existed between basal and hCG-stimulated levels of testosterone production. This was surprising as no significant difference in response to hCG was found between the low, medium and high testosterone

producing groups. However it does agree with the findings of Hsu *et al.* (1978) who found a very high positive correlation between specific hCG binding and intratesticular testosterone concentration in homogenised testicular tissue. Sharpe *et al.* (1980) found that LH receptor levels in the human testis are similar at 23-42 years and 70-72 years, again leading to the conclusion that the Leydig cells isolated by these techniques are functioning normally.

It was shown that the relative proportions of light and dark Leydig cells isolated by Percoll purification was an accurate reflection of the proportions found in the whole testis. This was a very important point to establish, it having been suggested previously that light Leydig cells may be less efficient testosterone producers, being more predominant in Leydig cell cultures which produce low amounts of testosterone (Simpson *et al.*, 1987). This study, in agreement with previous studies (Harman *et al.*, 1982; Paniagua *et al.*, 1987; Johnson *et al.*, 1988), found no correlation between testosterone production *in vitro* and factors such as age, paired testicular weight, and in the studies quoted, serum gonadotrophins and general testicular morphology. It was thus interesting to find that the 2 subjects used in this study who were of a similar age, with similar morphology and whose Leydig cells produced equivalent amounts of testosterone *in vitro,* also had very similar ratios of light: dark Leydig cells. As both the age and gross testicular morphology have been shown not to correlate with testosterone production *in vitro* or total testicular testosterone content, perhaps it is the ratio of light: dark Leydig cells in the testis which determines total testosterone output. This is also suggested by the study by Simpson *et al.* (1987) reported above.

It was not possible to separate light and dark Leydig cells by elutriation. Fractions containing cells with average diameters of 14, 16 and 19μM contained equal proportions of light: dark Leydig cells. The Leydig cells with the highest intrinsic testosterone producing ability were isolated in fraction 4, that is they formed the larger Leydig cell population. However they did not form a significant part of the total Leydig cell population (10% of the total). These cells were also the least responsive to hCG; it is possible that they were unresponsive because they are already working to their maximum capacity, an idea supported by the fact that the basal level of testosterone production by cells in this fraction was twice

the level of testosterone produced in response to hCG in either of the other 2 fractions. Thus no conclusion can be made from this experiment as to differential testosterone production by light and dark Leydig cells. Papadopoulos *et al.* (1987a) proposed the existence of 2 populations of Leydig cells based on the ability of cells isolated from different densities on Percoll gradients to differentially bind iodinated hCG. However, the densities at which these fractionated on Percoll (1.045-1.055 and 1.075g/ml) are in accord with the densities at which Leydig cells were isolated in this study and in the study by Simpson *et al.* (1987). This latter study showed no significant difference in either basal or hCG-stimulated testosterone production between Leydig cells in either layer. The results of the experiment reported here also argue against the existence of discrete populations of Leydig cells but in favour of a Leydig cell population that shows a normal bell-shaped distribution with respect to testosterone production.

In summary, the experiments presented in this chapter have shown that it is possible to isolate Leydig cells from ageing human testes that are responsive to stimulation by hCG, but there is a wide variation in the level of response between individuals. However studies in the literature suggest that such variation is also seen in younger populations. The ratio of light: dark Leydig cells isolated by Percoll purification was found to be a true representation of the ratio found in the intact testis. Finally it was shown that it is not possible to separate light and dark Leydig cells on the basis of size, and no conclusion could be made as to whether light and dark Leydig cells have different steroidogenic capacities.

5. Morphology of the human testis

The previous chapter described the existence of light and dark Leydig cells in the human testis. It was shown that the two populations could not be separated on the basis of size, making it impossible to reach a conclusion about possible differences in their steroidogenic capacity. The ability of human Leydig cells to secrete testosterone in *in vitro* cultures was shown to be highly variable. The studies presented in the present chapter used morphometric techniques to estimate the total number of Leydig cells as well as the number of light and dark Leydig cells in each testis. Other testicular cell types were also counted and the composition of each testis was calculated on a percentage volume basis. Correlations were made between various morphometric factors for each testis and the ability of Leydig cells isolated from that testis to produce testosterone *in vitro*, in an attempt to explain the variations observed in the preceding chapter.

5.1. Introduction

The ability of human Leydig cells to produce testosterone varies considerably between individuals. Such individual differences have been reported in studies which measured intratesticular testosterone content in whole testicular tissue fragments (Hirsh *et al.*, 1981; de la Torre *et al.*, 1982), in total testicular homogenates (Hsu *et al.*, 1978) or testosterone production by isolated human Leydig cells (see previous chapter and Simpson *et al.*, 1987). Several studies have tried to explain these variations by relating *in vitro* testosterone production to factors such as age, paired testicular weight, serum gonadotrophins and general testicular morphology (Harman *et al.*, 1982; Paniagua *et al.*, 1987; Johnson *et al.*, 1988), with no success. At the same time it is also well recognised that the population of Leydig cells found in the human testis is highly heterogeneous (Schulze, 1984; Paniagua *et al.*, 1987; Simpson *et al.*, 1987). In particular the existence of variably staining Leydig cells has been described, in both perfused and immersion-fixed tissue (Schulze, 1984; Simpson *et al.*, 1987). Simpson *et al.* (1987) proposed that there might be inherent differences in the steroidogenic ability of light and dark Leydig

cells, but to date this possibility has not been explored. Therefore the aim of this study was to use morphometric analysis of testes from a number of men to discover whether there was any relationship between the relative number of light and dark Leydig cells and the subsequent steroidogenic capacity of Leydig cells isolated from these testes.

5.2. Experimental Procedures

5.2.1. Histology

Testicular tissue from random positions was processed as described in Chapter 3. Sections of 1μM thickness were stained with toluidine blue and examined and photographed using a Zeiss photomicroscope.

5.2.2. Random sampling

Fields were selected at random to ensure that all morphometric analysis was performed on unbiased and representative samples.

5.2.3. Determination of average Leydig cell nuclear diameter

The average diameter of both light and dark Leydig cells in individual testes was measured using a Plan Apo 60 x oil objective fitted to a Leitz microscope connected to the imagan2 (Kompira Ltd., Strathclyde) interactive image analysis system for IBM computers. In every case 25 light and 25 dark Leydig cells were measured in sections from three different areas of the testis, to give a total of 75 measurements of nuclear diameter for both light and dark Leydig cells for every man.

5.2.4. Determination of Leydig cell number

The number of light and dark Leydig cell nuclei in individual testes was calculated using the Floderus equation (Floderus, 1944);

$$N_V = N_A / D + 2h$$

where N_V = numerical density, or, the number of light or dark Leydig cells per unit volume of the testis

N_A = average number of light or dark Leydig cell nuclei in a known area

D = average nuclear diameter of light or dark Leydig cells

h = height of the smallest visible nuclear cross-section, assumed to be $^1/_{10}$th of D (Wing and Christensen, 1982)

To determine N_A, Leydig cell nuclei were counted in 10 randomly selected microscopic fields (100,000μm^2) in 3 sections taken from different areas of the testis. Measurements were performed using a Zeiss microscope fitted with a Plan Apo x 20 oil objective.

The number of light and dark Leydig cells in each testis was calculated by multiplying N_V by the volume of the testis. It has been shown previously that the specific gravity of human testicular tissue approximates to unity (Barbara Simpson, PhD thesis, 1987). It was therefore assumed that testicular weight was interchangeable with testicular volume (i.e. 1g = 1cm^3). It is recognised that all fixed, embedded tissue blocks shrink during the processing procedure, so that the fresh weight of the testis is greater than the weight after fixation. However, if the degree of shrinkage is assumed to be uniform between samples, then it is widely accepted that the total volume of the testis can be estimated by equating with weight. Mendis-Handagama & Ewing (1990) demonstrated that in normal rat testes this assumption had negligible effects on the estimates of Leydig cell numbers per testis.

5.2.5. Testicular composition

The composition of each testis was determined using a 100,000 μm^2 graticule. The cell type or structure beneath every intersection point on the graticule (121 points) was recorded in 10 fields in sections taken from three different areas of each testis. The mean number of counts for each item was determined and then expressed as a percentage of the total counts possible. The volume per testis was calculated by multiplying this by testicular volume. In this way the proportion of testicular tissue comprising the following structures was assessed. Factors scored were,

germ cells, interstitium, seminiferous tubule lumen, vacuoles and fat droplets in the seminiferous epithelium, blood vessels, peritubular tissue and the cytoplasm of light and dark Leydig cells. Different cell types were recognised using the criteria defined by Holstein & Roosen-Runge (1987). Light and dark Leydig cells were distinguished on the basis of their differential staining with toluidine blue.

5.2.6. Relationships between cell types in the testis

The statistical relationships between various testicular cell types were determined by simple regression analysis. In this way possible relationships between cell types and testosterone production by isolated Leydig cells, responsiveness of isolated Leydig cell to stimulation by a supramaximal dose of hCG, age and testicular weight were also assessed.

5.3. Results

5.3.1 Determination of average Leydig cell nuclear diameter

The average nuclear diameter of light and dark Leydig cells did not differ significantly from each other, nor were there significant differences in light or dark nuclear diameter between individuals. The average nuclear diameter of a light Leydig cell nucleus was 6.96±0.4μm (n=25) and the average nuclear diameter of a dark Leydig cell nucleus was 6.76±0.38μm (n=25). These values were then used in the Floderus equation which calculates the total number of light and dark Leydig cells in each testis.

5.3.2. Determination of Leydig cell number

The calculated number of light and dark Leydig cells per testis for each individual is shown in Table 5.1. The table also shows the total number of Leydig cells per testis and the ratio of light : dark Leydig cells in that testis. The number of Leydig cells was found to correlate significantly with testicular weight (p <0.001), or simply, larger testes contain more Leydig

cells. Although there was a decline in the total number of Leydig cells with age, this was not statistically significant (p <0.09; Figure 5.1.A), and this decline was reflected in reductions in both light and dark Leydig cells with age (Figure 5.1.B and C). No correlation was found between basal testosterone production by isolated Leydig cells and the total number of Leydig cells (Figure 5.2.A), nor were the numbers of either light or dark Leydig cells shown to be related to subsequent basal testosterone production (Figure 5.2.B and C). The ratio of light to dark Leydig cells was also not correlated to testosterone production (Figure 5.2.D). The response of isolated Leydig cells to hCG, represented by the fold increase in testosterone production upon the addition of hCG, was also unrelated to the 4 measurements of total and relative Leydig cell populations (Figure 5.3., see also Table 5.3.).

In chapter 4, human Leydig cell preparations were classified into 3 groups according to their ability to produce testosterone *in vitro* (low, medium and high producers). These divisions represent different capacities to produce testosterone, as each refers to low, medium or high production per 10^6 cells. Figure 5.4 shows the average number of Leydig cells measured in each of these groups. The total number of Leydig cells per testis increased from the low to medium to high groupings, with the number of Leydig cells in the high capacity group being significantly greater than that measured in the low capacity group (p <0.05). The numbers of light and dark Leydig cells making up the total Leydig cell population in each group is shown in Figure 5.5. There was no significant difference in the number of light or dark Leydig cells within any group. While there was no significant difference in the number of dark Leydig cells between groups, the number of light Leydig cells was significantly greater in the high testosterone production group when compared to the low group (p <0.05). The significance of the ratio of light : dark Leydig cells and the capacity to produce testosterone was also considered. The ratio was significantly lower in the medium group when compared with the low group (p <0.01, Figure 5.6.)

Table 5.1. Composition of the Leydig cell population in individual testes.

The total number of Leydig cells in each testis was calculated using the Floderus equation. The numbers of light and dark Leydig cells and the ratio of light to dark Leydig cells are also given.

Patient no.	number of Leydig cells/testis (x 10^6) light	dark	total	ratio light : dark
1	144.61	206.66	351.28	1:1.43
2	13.75	36.68	50.42	1:2.66
3	70.43	69.02	139.45	1:0.98
4	49.73	100.16	149.89	1:2.01
5	52.54	39.86	92.39	1:0.76
6	30.24	44.53	74.77	1:1.47
7	43.11	34.23	77.34	1:0.79
8	78.93	35.79	114.71	1:0.45
9	112.28	153.68	265.97	1:1.37
10	96.15	81.51	177.66	1:0.85
11	60.74	65.44	126.18	1:1.08
12	69.48	90.35	159.83	1:1.3
13	19.55	23.67	43.2	1:1.21
14	156.14	122.84	278.98	1:0.79
15	19.94	23.58	43.53	1:1.18
16	120.6	86.12	206.72	1:0.71
17	31.46	24.2	55.66	1:0.77
18	98.51	89.7	188.21	1:0.91
19	27.37	46.34	73.71	1:1.69
20	30.61	48.53	79.14	1:1.59
21	92.18	76.28	168.46	1:0.83
22	106.59	85.57	192.16	1:0.81
23	41.42	50.34	91.76	1:1.21

Figure 5.1. Correlation between the number of Leydig cells per testis and age.

A. Correlation between total Leydig cell population *vs.* age, r = 0.349, $p < 0.09$.

B. Correlation between the number of light Leydig cells per testis *vs.* age, r = 0.36.

C. Correlation between the number of dark Leydig cells per testis *vs.* age, r = 0.368.

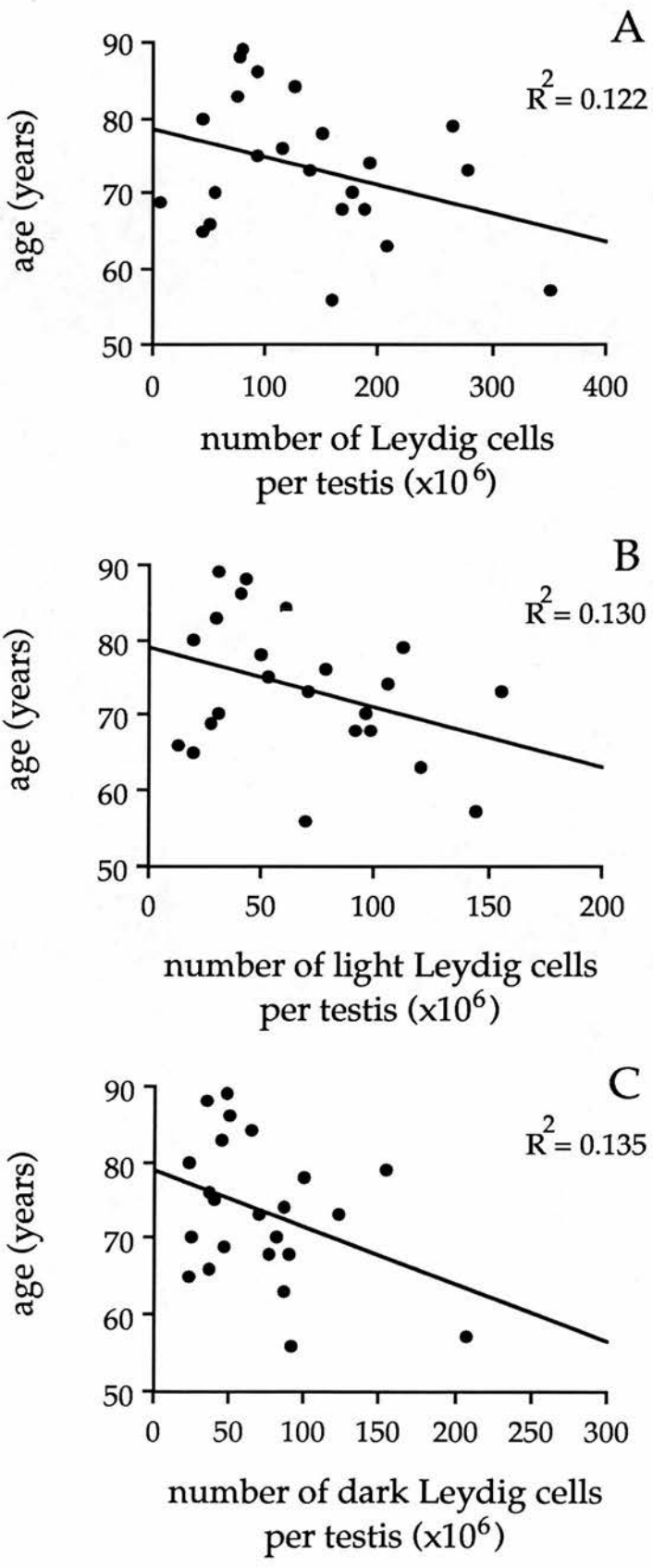

A
$R^2 = 0.122$
age (years)
90
80
70
60
50
0
100
200
300
400
number of Leydig cells
per testis (x10^6)
B
$R^2 = 0.130$
age (years)
90
80
70
60
50
0
50
100
150
200
number of light Leydig cells
per testis (x10^6)
C
$R^2 = 0.135$
age (years)
90
80
70
60
50
0
50
100
150
200
250
300
number of dark Leydig cells
per testis (x10^6)

Figure 5.2. Correlation between the number of Leydig cells per testis and basal testosterone production *in vitro*.

A. Correlation between total Leydig cell population *vs.* testosterone production, r = 0.287. **B.** Correlation between the number of light Leydig cells per testis *vs.* testosterone production, r = 0.229. **C.** Correlation between the number of dark Leydig cells per testis *vs.* testosterone production, r = 0.275. **D.** Correlation between the ratio of light : dark Leydig cells per testis *vs.* testosterone production, r = 0.139.

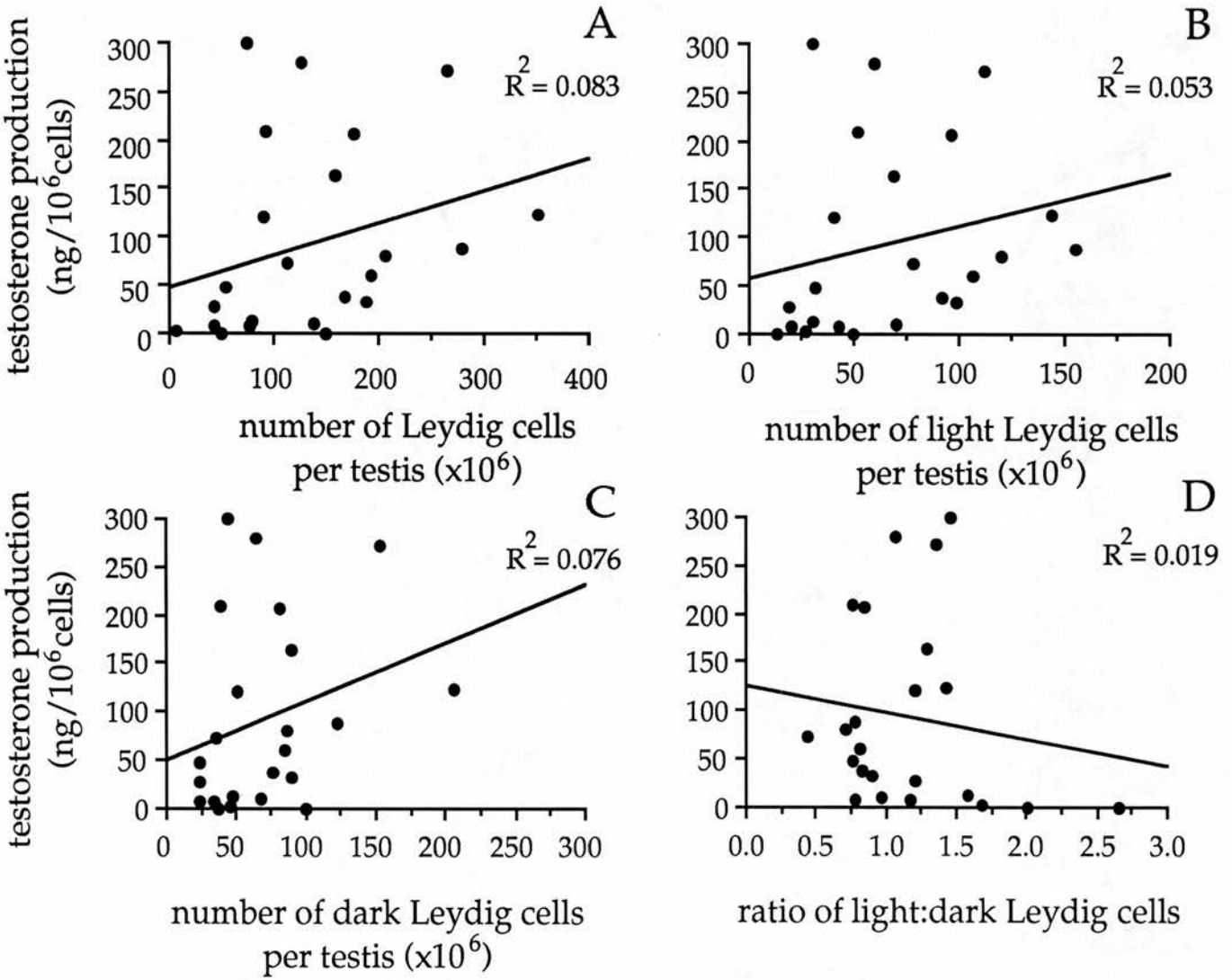

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
R^2 = 0.083
R^2 = 0.053
R^2 = 0.076
R^2 = 0.019
number of Leydig cells per testis (x10^6)
number of light Leydig cells per testis (x10^6)
number of dark Leydig cells per testis (x10^6)
ratio of light:dark Leydig cells
0
50
100
150
200
250
300
400
0.0
0.5
1.0
1.5
2.0
2.5
3.0

Figure 5.3. Correlation between the number of Leydig cells per testis and the fold response to hCG of isolated Leydig cells.

A. Correlation between total Leydig cell population *vs.* fold response, r = 0.019. **B.** Correlation between the number of light Leydig cells per testis *vs.* fold response, r = 0.046. **C.** Correlation between the number of dark Leydig cells per testis *vs.* fold response, r = 0.037. **D.** Correlation between the ratio of light : dark Leydig cells per testis *vs.* fold response, r = 0.008.

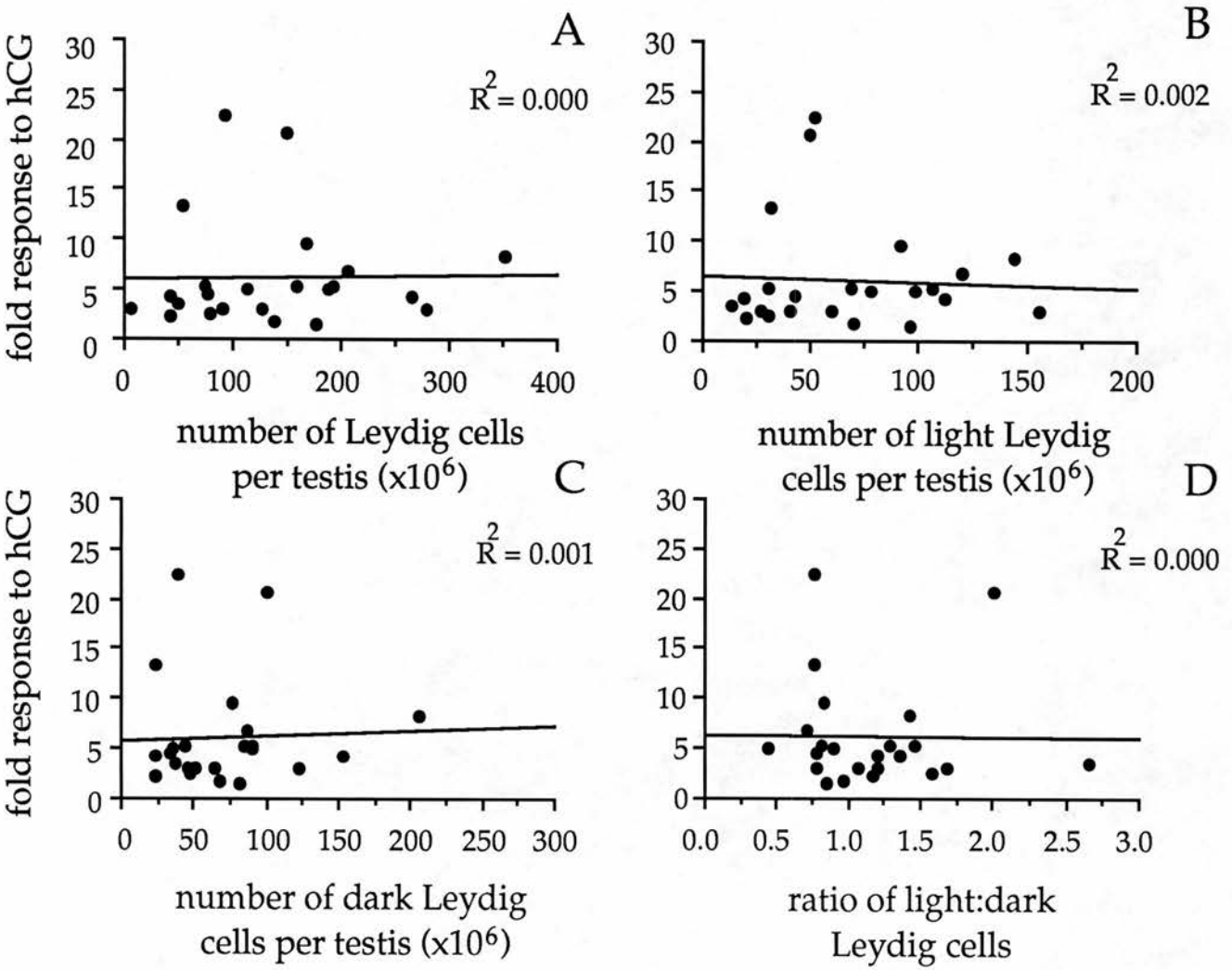

A
B
C
D
fold response to hCG
fold response to hCG
R^2 = 0.000
R^2 = 0.002
R^2 = 0.001
R^2 = 0.000
number of Leydig cells per testis (x10^6)
number of light Leydig cells per testis (x10^6)
number of dark Leydig cells per testis (x10^6)
ratio of light:dark Leydig cells

Figure 5.4. The capacity of isolated Leydig cells to produce testosterone *in vitro* (low, medium or high, ng/10^6 cells) and the total number of Leydig cells per testis in each group.

Each column is the mean ±S.D. for 5 testes. *p <0.05, significantly different from the low group.

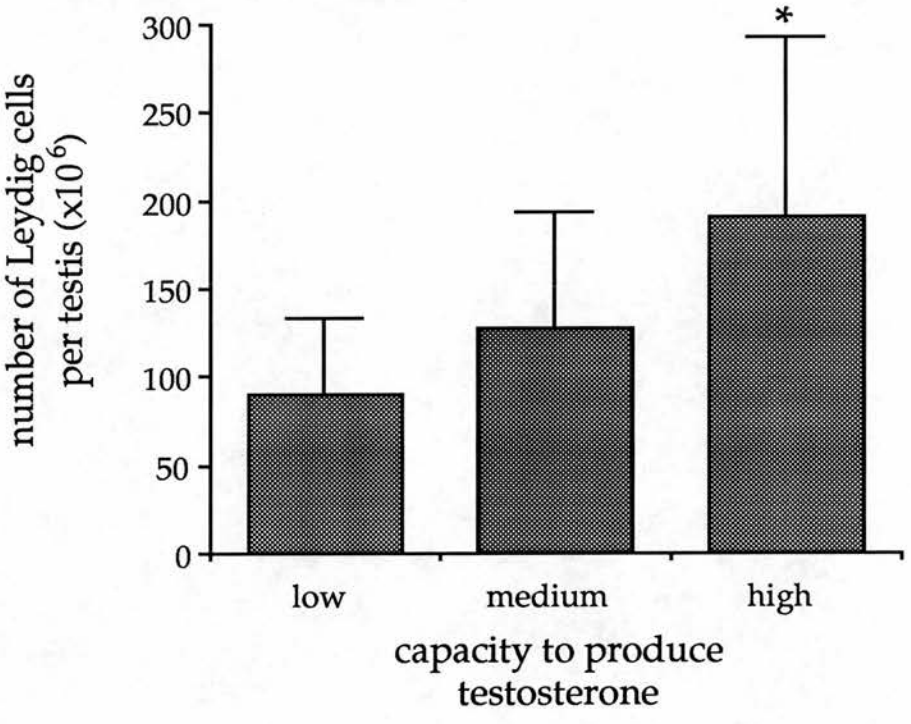

number of Leydig cells per testis (x10^6)
300
250
200
150
100
50
0
*
low
medium
high
capacity to produce testosterone

Figure 5.5. The capacity of isolated Leydig cells to produce testosterone *in vitro* (low, medium or high, ng/10^6 cells) and the number of light and dark Leydig cells per testis in each group.

Each column is the mean ±S.D. for 5 testes. *p <0.05, significantly different from the number of light Leydig cells in the low group.

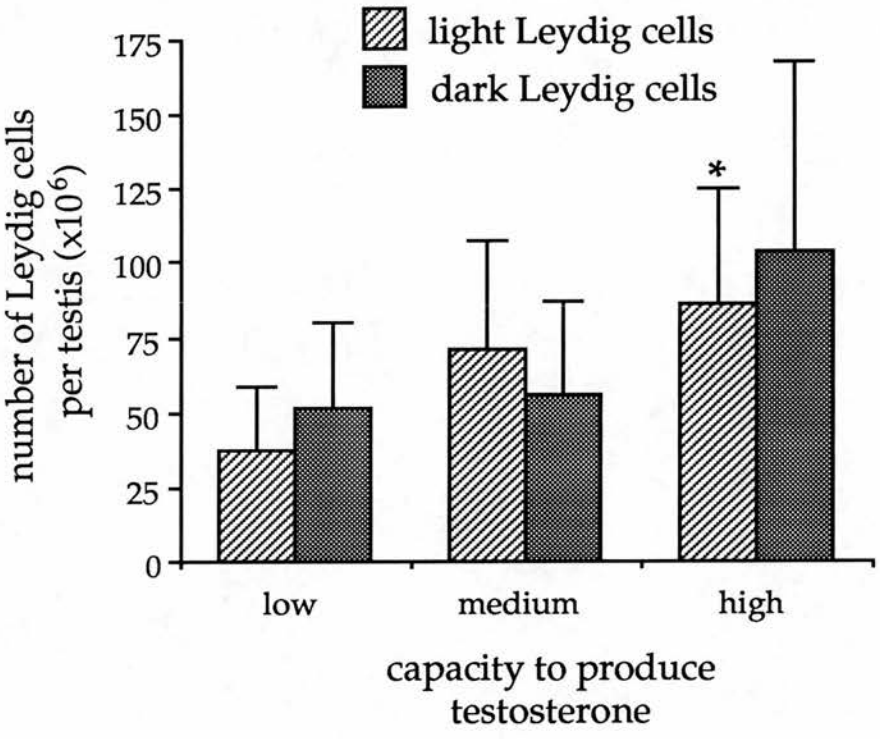
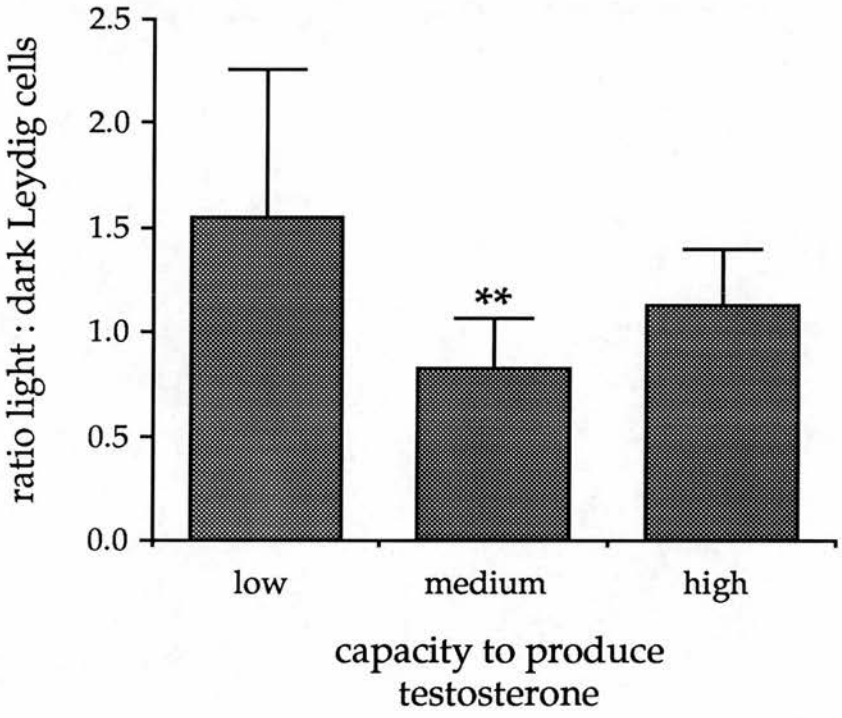

light Leydig cells
dark Leydig cells
number of Leydig cells per testis (x10⁶)
175
150
125
100
75
50
25
0
*
low
medium
high
capacity to produce testosterone

Figure 5.6. The capacity of isolated Leydig cells to produce testosterone *in vitro* (low, medium or high, ng/10^6 cells) and the ratio of light to dark Leydig cells per testis in each group.

Each column is the mean ±S.D. for 5 testes. $^{**}p < 0.01$, significantly different from the Leydig cell ratio in the low group.


ratio light : dark Leydig cells
2.5
2.0
1.5
1.0
0.5
0.0
**
low
medium
high
capacity to produce testosterone

5.3.3. Testicular composition

The average percentage contribution of each factor to the total volume of the testis is shown in Table 5.2. The minimum and maximum values are also given. Considering the variation in *in vitro* testosterone production by Leydig cells isolated from these testes, there is remarkable consistency in their make-up. In parallel with this finding, the percentage contribution of each factor measured had a positive and significant correlation with testicular weight (see Table 5.3.).

5.3.4. Relationships between cell types in the testis

Regression analysis was calculated for every possible pairing of cell types, and the correlation coefficients obtained are presented in Table 5.3. As discussed above, testicular weight showed a positive correlation with all testicular factors measured. Neither basal testosterone production by isolated Leydig cells nor the fold response of these cells to hCG correlated with any of the structural parameters measured. However, significant correlations amongst several of these parameters were found. The volume of light Leydig cell cytoplasm correlated with the volume of light Leydig cell nuclei, and ditto for dark Leydig cell nuclei and cytoplasm. However the correlations of cytoplasm and nuclei with other factors measured were only comparable in dark Leydig cells and were considerably different for light Leydig cells. These results are summarised in Table 5.4. Dark Leydig cell nuclei and cytoplasm were shown to correlate significantly with the volume of germ cells and tubule lumen (p <0.001) and with the volume of fat droplets in the seminiferous epithelium and the volume of the interstitial space (p <0.01). There was no correlation with blood vessel volume, peritubular tissue or the number of vacuoles in the seminiferous epithelium. The correlations seen with light Leydig cells were quite different. Light Leydig cell nuclei showed a relatively low correlation with germ cell volume (p <0.01) but did not correlate with any other factor. Light Leydig cell cytoplasm showed strong correlations with the volumes of germ cells, the interstitium, blood vessels, peritubular tissue and the number of fat droplets in the seminiferous epithelium (p <0.001). The correlation with

the number of vacuoles in the seminiferous epithelium was less strong (p <0.01) and there was no correlation between light Leydig cell cytoplasm and the volume of the tubular lumen. Besides showing a positive correlation with both light and dark Leydig cell volumes, germ cell volume correlated positively with all of the other testicular structures measured. Without considering Leydig cells, in general positive correlations existed between most of the testicular structures measured (see Table 5.3.). Photomicrographs of testes with different ratios of light: dark Leydig cells are shown in Figure 5.4.

Table 5.2. Composition of the testis in 26 men.

Data represent the average ± S.D. percentage volume occupied by each component.

Testicular component	volume of testis occupied (%)
light Leydig cell cytoplasm	0.99 ± 0.49
light Leydig cell nuclei	0.41 ± 0.26
dark Leydig cell cytoplasm	1.32 ± 0.64
dark Leydig cell nuclei	0.38 ± 0.22
germ cells	41.81 ± 9.41
interstitium	19.49 ± 6.45
blood vessels	2.02 ± 0.69
peritubular tissue	22.44 ± 7.44
tubular lumen	3.55 ± 2.36
epithelial vacuoles	4.23 ± 2.09
fat droplets	3.37 ± 1.2

Table 5.3. Correlation Coefficients of testicular parameters.

Correlations between the volumes/testis of all testicular components measured are shown. Figures in bold represent p **<0.001**, figures in italics represent p *<0.01*. N.S. = not significant.

Abbreviations used : basal T = basal testosterone production (ng/10^6 cells); fold = fold response to a maximally-stimulating dose of hCG; LLCN = light Leydig cell nuclei; LLCC = light Leydig cell cytoplasm; DLCN = dark Leydig cell nuclei; DLCC = dark Leydig cell cytoplasm; intstm = interstitium; bv = blood vessels.

	weight	basal T	fold	LLCN	LLCC	DLCN	DLCC	germ cells	intstm	b.v.	PT	lumen	vacuoles	fat drops
weight	—	0.01	0.07	**0.50**	**0.81**	**0.64**	**0.70**	**0.97**	**0.87**	**0.88**	**0.90**	**0.75**	*0.58*	**0.76**
basal T	0.01	—	0.02	0.04	0.06	0.31	0.34	0.01	0.02	0.13	0.04	0.17	0.09	0.01
fold	0.07	0.02	—	0.00	0.13	0.10	0.05	0.01	0.01	0.17	0.03	0.02	0.20	0.26
LLCN	**0.50**	0.04	0.00	—	**0.80**	N.S.	N.S.	*0.59*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
LLCC	**0.81**	0.06	0.13	**0.80**	—	N.S.	**0.69**	**0.83**	**0.62**	**0.65**	**0.70**	N.S.	*0.52*	**0.70**
DLCN	**0.64**	0.31	0.10	N.S.	N.S.	—	**0.88**	**0.63**	*0.55*	N.S.	N.S.	**0.75**	N.S.	*0.53*
DLCC	**0.70**	0.34	0.05	N.S.	**0.69**	**0.88**	—	**0.70**	*0.61*	N.S.	N.S.	**0.64**	N.S.	*0.54*
germ cells	**0.97**	0.01	0.01	*0.59*	**0.83**	**0.63**	**0.70**	—	**0.80**	**0.79**	**0.81**	**0.67**	*0.59*	**0.73**
intsm	**0.87**	0.02	0.01	N.S.	**0.62**	*0.55*	*0.61*	**0.80**	—	**0.79**	**0.72**	**0.70**	N.S.	*0.57*
b.v.	**0.88**	0.13	0.17	N.S.	**0.64**	N.S.	N.S.	**0.79**	**0.79**	—	**0.92**	**0.80**	*0.45*	*0.64*
PT	**0.90**	0.04	0.27	N.S.	**0.70**	N.S.	N.S.	**0.81**	**0.72**	**0.92**	—	**0.70**	*0.61*	**0.75**
lumen	**0.75**	0.17	0.02	N.S.	N.S.	**0.76**	**0.64**	**0.67**	**0.70**	**0.80**	**0.70**	—	N.S.	*0.60*
vacuoles	0.58	0.09	0.20	N.S.	*0.52*	N.S.	N.S.	*0.59*	N.S.	*0.45*	*0.61*	N.S.	—	*0.53*
fat drops	**0.76**	0.01	0.26	N.S.	**0.70**	*0.53*	*0.54*	**0.73**	*0.58*	*0.64*	**0.75**	*0.60*	*0.53*	—

Table 5.4. Correlations of light and dark Leydig cell nuclei and cytoplasm with other components of the testis.

./ ./= p <0.001

./ = p <0.01

x = no significant correlation

	light nuclei	light cytoplasm	dark nuclei	dark cytoplasm
germ cells	✓	✓✓	✓✓	✓✓
interstitium	✗	✓✓	✓	✓
blood vessels	✗	✓✓	✗	✗
peritubular tissue	✗	✓✓	✗	✗
lumen	✗	✗	✓✓	✓✓
vacuoles	✗	✓	✗	✗
fat droplets	✗	✓✓	✓	✓

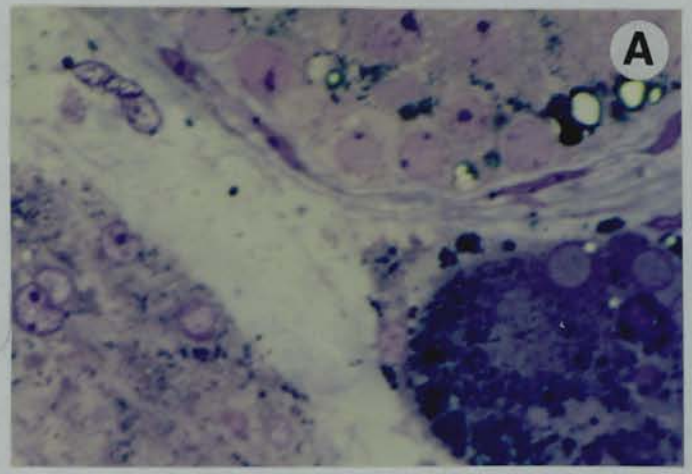
Figure 5.4. Histology of the human testis.

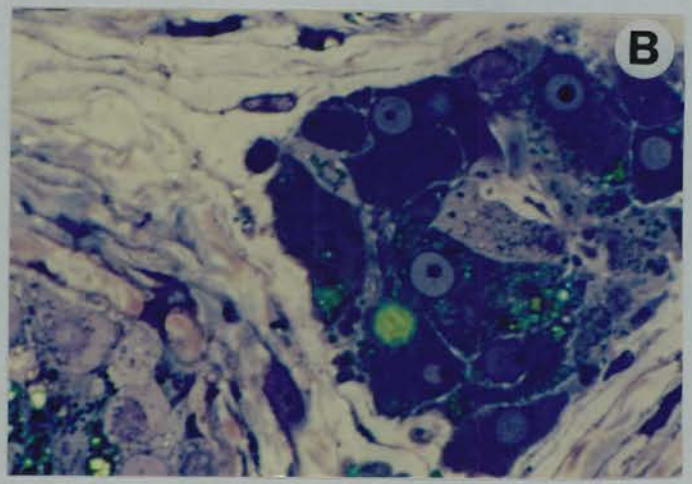
Examples of testicular morphology in testes where the ratio of light : dark Leydig cells was : **A.** 1: 0.45

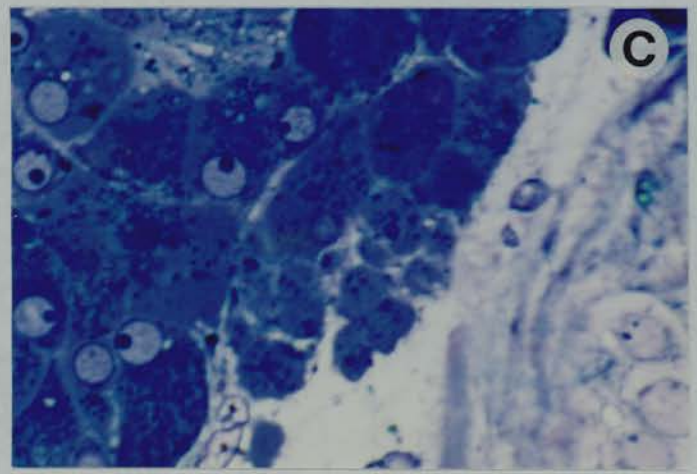
B. 1: 0.98

C. 1: 1.69

D. 1: 2.66


A


B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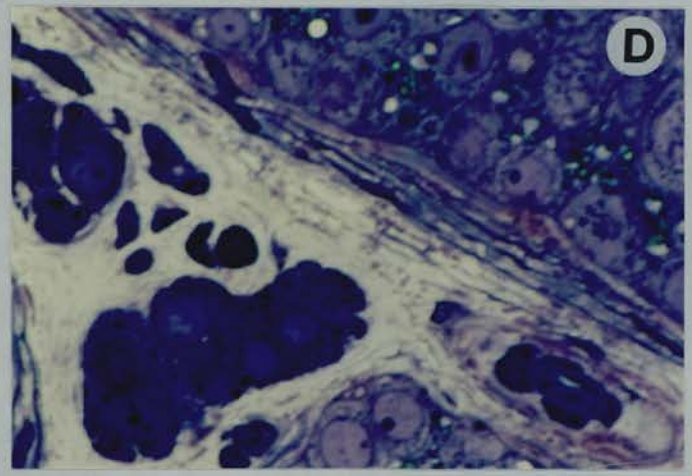

D

5.4. Discussion

The objective of the morphometric analysis described in this chapter was to accumulate accurate morphometric information about the human testis to allow correlations to be made between physiological and biochemical findings in order to identify potentially important cell-cell interactions in the human testis.

The average diameter of a Leydig cell nucleus was 6.86±0.39μm and there was no difference in nuclear diameter between the light and dark subpopulations of Leydig cells. The average number of Leydig cells per testis was 139.12±81.89 x 10^6, with minimum and maximum values of 43.2 x 10^6 and 351.28 x 10^6 respectively. The number of light and dark Leydig cells per testis is shown in Table 5.1. The numbers of Leydig cells per testis is in agreement with the studies by Neaves *et al.*, (1984), who found a range of 159-330 x 10^6 Leydig cells per testis in men aged between 50-76 years. Similar numbers were reported by Paniagua *et al.*, (1987) who found that the number of Leydig cells per testis in men aged 51-90 years varied between 122-382 x 10^6. Neaves *et al.*, (1984) described a 44% reduction in the number of Leydig cells when comparing a group of men aged 50-76 years with a group aged 20-48 years. A similar, though statistically insignificant decline in the number of Leydig cells with increasing age was seen in this study. The number of Leydig cells per testis showed a positive correlation with testicular weight (p <0.001), in fact there was a positive correlation between testicular weight and all parameters measured (see Table 5.3.).

No correlation was found between the total number of Leydig cells per testis and the amounts of testosterone produced by those Leydig cells when isolated and cultured. As described in chapter 4, a marked individual variation was found in the amounts of testosterone produced by a unit number (10^6) of Leydig cells. Hirsh *et al.* (1981) were also unable to demonstrate a significant correlation between the volume densities of interstitial cells and the intratesticular testosterone content measured in homogenized testicular fragments. The lack of correlation between the amounts of testosterone produced per 10^6 Leydig cells *in vitro* and the total number of Leydig cells per testis, shows that Leydig cells isolated from small testicular populations were neither more nor less able to

produce testosterone than Leydig cells isolated from a larger population. This finding refutes the idea proposed by Neaves *et al.* (1984) that Leydig cells from small testicular populations have to work harder than those from larger populations in order to maintain the intratesticular levels of testosterone necessary to drive spermatogenesis. Neaves *et al.* (1985) described how Leydig cells in the human testis are subject to degeneration and dissolution with increasing age. It is thus possible that light and dark Leydig cells represent one population of steroid synthesising cells and a second population of cells which are inactive due to either degeneration, or are inactive during regeneration to replace phagocytosed Leydig cells. To address these points and to examine more closely the correlation between *in vitro* testosterone production and Leydig cell numbers, correlations were made between the number of light or dark Leydig cells in the testis and testosterone production per 10^6 isolated Leydig cells. No correlation was found when this subdivision of the Leydig cell population was made.

Isolated Leydig cells were subdivided into 3 groups (low, medium and high) based on the amount of testosterone produced per 10^6 cells per 20h in culture. In general the capacity of Leydig cells to produce testosterone *in vitro* increased in concert with the size of the *in vivo* Leydig cell population. This contradicts the proposal by Neaves *et al.* (1984), described above, that Leydig cells from small populations work harder than those from larger populations. In fact the opposite appears to be true, with Leydig cells from large populations producing more testosterone *in vitro* than Leydig cells from smaller populations. The increase in Leydig cell numbers found between the low, medium and high groups was matched by increases in the numbers of both light and dark Leydig cells. When the ratio of light : dark Leydig cells in these groups was considered, on average, testes in the low and high groups both had more dark than light Leydig cells (ratio light : dark 1 : 1.55±0.71 and 1 : 1.125±0.27, respectively). In the medium group, there were, on average, more light than dark Leydig cells (1 : 0.83±0.24). As Leydig cells with both low and high testosterone producing capacities had a higher proportion of light than dark Leydig cells, it is not possible to equate the capacity or inability to produce testosterone *in vitro* with the predominance of either subpopulation of Leydig cells. In fact, in the majority of men (13/23) the

ratio of light : dark Leydig cells was not significantly different from 50 : 50. There was also a positive correlation (p <0.001) between light and dark Leydig cell cytoplasmic volume indicating that larger populations of Leydig cells contain more of both Leydig cell types. The available evidence does not support the hypothesis that light and dark Leydig cells represent steroidogenically active and inactive subpopulations. As there is no evidence to the contrary it has been assumed that both light and dark Leydig cells are able to produce testosterone.

The ability of isolated Leydig cells to respond to hCG was represented by the fold increase in testosterone production when comparing basal testosterone production with the amount of testosterone produced by Leydig cells exposed to a maximally stimulating dose of hCG. No correlation was found between the fold response to hCG and either total Leydig cell number, the number of light and dark Leydig cells nor the ratio of light to dark Leydig cells. It can therefore be stated that light and dark Leydig cells cannot be differentiated on the basis of subsequent testosterone production, neither does it appear that one subtype is more responsive to the effects of hCG than the other. On the basis of these correlations it is not possible to make a functional distinction between light and dark Leydig cells.

As no correlations could be discerned between physical and biochemical factors, the study went on to examine the relationship between several intratesticular cell types and structures. Point counting was undertaken in order to determine the volume of testis occupied by each factor considered. The percentage composition of the testis was remarkably similar between individuals (Table 5.2.). This suggested that a biochemical, rather than a physical factor was important when considering the variation in testosterone production by Leydig cells isolated from these testes. It was hoped that correlations between intratesticular cell types and structures would allow some insight into the source and /or nature of this factor.

Both the nuclear and cytoplasmic volume of dark Leydig cells were found to correlate with the same testicular structures and to the same degree (see Table 5.4.). However, the volumes of light nuclei and cytoplasm, although correlating with each other, did not show parallel correlations with other testicular structures. It is possible that this is a

reflection of the point counting system, as qualitatively, dark Leydig cells appear to have cytoplasm of a uniform, regular size, while the cytoplasm of light Leydig cells seems to be of more variable dimensions. Thus the number of counts of dark Leydig cell cytoplasm would be relatively consistent between sections whilst the number of counts of light Leydig cell cytoplasm would be more variable, and so correlate less clearly with the counts of light Leydig cell nuclei. A relationship between the steroidogenic ability of Leydig cells and their content of smooth endoplasmic reticulum (SER) has been demonstrated (Ewing & Zirkin, 1983), and when correlations were made between Leydig cell volume and other testicular cell types, it was assumed that cytoplasmic rather than nuclear correlations were of greater importance. After having been able to draw no functional distinctions between light and dark Leydig cells on the basis of correlations with *in vitro* testosterone production or fold response to hCG, it was interesting to find that light and dark Leydig cell cytoplasmic volume correlated with different testicular cell types and structures. Both correlated with germ cell volume, which might be expected as testosterone drives spermatogenesis (Steinberger, 1971). Both also correlated with the volume of the interstitium and with the volume of seminiferous epithelium taken up by fat droplets, although the correlation was strongest for light rather than dark Leydig cell cytoplasm ($p < 0.001$ *vs* $p < 0.01$). The volume of light Leydig cell cytoplasm alone correlated with the volume of peritubular tissue and the volume of interstitium occupied by blood vessels. Only the volume of dark Leydig cell cytoplasm correlated with the volume of the tubular lumen. These differential correlations could perhaps be the first indications of a division of responsibilities between light and dark Leydig cells.

Considering first the correlations shown by light Leydig cells only, can rational explanations for these correlations be given? If it is accepted that the more active a cell is, the more demand it exerts on energy supply, then a relationship between Leydig cell volume and blood vessel volume might exist. Alternatively, Bergh & Damber (1992) demonstrated that arterioles have androgen receptors and thus testosterone can be presumed to have effects on the vasculature. Increasing thickness of the peritubular wall is possibly a barrier to the transport of testosterone from the interstitium into the seminiferous epithelium (Johnson, 1986). Thus the

thicker the peritubular wall, perhaps the greater the amount of testosterone needed to produce the high local concentrations that drive spermatogenesis. While the volume of dark Leydig cell cytoplasm did not correlate with these factors it did show a correlation with tubular lumen volume. In their morphometric study of the rat testis, Wing & Christensen (1982) describe variations in the size of the lumen according to the stage of the spermatogenic cycle. There is a significant increase in the volume of the lumen between stages V-VIII, followed by a significant decrease in luminal volume after spermiation. The production of seminiferous tubule fluid is testosterone-dependent (Jegou, 1983), so perhaps this correlation is not surprising, although if this explanation is accepted, it is not clear why the correlation is found with dark Leydig cells only. Although the spatial organisation of the various stages of spermatogenesis is not directly comparable between rat and man (Schulze & Rehder, 1984), it is possible that lumen size in man could also vary in accordance with the spermatogenic stage. As this study has not attempted to distinguish germ cell types, lumen size is the only factor measured which could possibly be related to the stage of spermatogenesis. Thus this correlation might be indicative of a dialogue between the seminiferous epithelium and dark Leydig cells.

Based on the different correlations observed between light and dark Leydig cells and other testicular cell types, it is possible to postulate different roles for light and dark Leydig cells. For example, one possible interpretation of these findings is that a dialogue between the seminiferous epithelium (probably via Sertoli cells) and Leydig cells does exist, that it is related to the stage of the spermatogenic cycle, and is mediated through dark rather than light Leydig cells. Thus while both light and dark Leydig cells may be active testosterone producers, perhaps only dark Leydig cells are responding directly to signals from the seminiferous epithelium. Evidence does exist to support the hypothesis of paracrine communication across the seminiferous epithelium of the human testis into the interstitium. Verhoeven & Cailleau (1987) demonstrated that cultured fragments of human seminiferous tubules produce a factor(s) able to stimulate testosterone production by isolated human Leydig cells. The authors speculated that such stimulatory factors may act as paracrine regulatory molecules. Papadopoulos (1991)

demonstrated that cultures of human Sertoli cells secrete a 79kDa protein that is able to stimulate steroidogenesis by human Leydig cells. The stimulatory effect of conditioned medium from human seminiferous tubule cultures on testosterone production by isolated Leydig cells is confirmed in studies described later in this thesis (chapter 9).

Bergh (1983, 1985) suggested the possibility of a Leydig cell cycle in the rat, paralleling the spermatogenic cycle. It was demonstrated that Leydig cells in the vicinity of stage VII-VIII tubules were significantly larger than Leydig cells adjacent to tubules at other stages. Stages VII-VIII have been shown to be androgen-dependent (Sharpe *et al.*, 1988; Sharpe *et al.*, 1992). In a later study, Fouquet (1987), although unable to show variations in Leydig cell profile area according to the stage of the seminiferous epithelium of adjacent tubules, did find that SER content was significantly higher when Leydig cells were adjacent to tubules at stages VI-VIII compared to those at XI-XII. However, the study was unable to correlate Leydig cell ultrastructure with the stages of the seminiferous epithelium in adjacent tubules in the adult monkey (*Macaca fascicularis*). There is some evidence for the existence of a Leydig cell cycle in the human testis. Regadera *et al.* (1991) found that Leydig cells in the testes of a 27 year old man exhibited different degrees of staining affinity for an anti-testosterone antibody. As only 30% of morphologically normal Leydig cells stained intensely for testosterone, and 10% showed no staining at all, the authors suggested the differences could be due to cyclical changes in the functional activity of Leydig cells.

It is therefore possible that a Leydig cell cycle exists in the human testis which is controlled by factors secreted from the seminiferous epithelium and that these signals are mediated through dark rather than light Leydig cells. The influence of the seminiferous epithelium on the remaining cells of the testis is suggested by the finding that of all the cell types and structures considered, it is germ cell volume alone that correlates significantly with all other factors measured. The idea that germ cells have a paracrine function is gaining wider acceptance. Accumulating evidence suggests that particular germ cell types can modulate Sertoli cell secretory function (see succeeding chapters, also Sharpe, 1992 and Jegou *et al.*, 1992 for review). For example, Allenby *et al.* (1991) demonstrated that the secretion of inhibin by rat seminiferous

tubules *in vitro* was regulated by elongate spermatids. Reciprocal influences also exist, in the rat it has been shown that testosterone exerts important effects on tubular protein secretion (Sharpe *et al.*, 1992). Testosterone also acts on peritubular cells to stimulate the secretion of P-Mod-S, which in turn modulates Sertoli cell function, at least *in vitro*. Thus the cell-cell communication that occurs in the rat testis is complex and is not limited to two cell interactions. Onoda *et al.* (1991) demonstrated that the secretion of a Sertoli cell protein(s) that stimulated Leydig cell steroidogenesis was modulated by the presence or absence of pachytene spermatocytes. Thus, in the rat, the modulatory influence of germ cells on other testicular cell types is becoming more clear. The results of the present study, in which germ cell volume correlated with all other testicular components, suggests that germ cells may have a similarly important role in the human testis.

In conclusion, the studies described in this chapter have confirmed that the number of Leydig cells present in the human testis declines with age. No correlation was found between the number of Leydig cells per testis and the ability of these Leydig cells to produce testosterone when isolated and cultured. This demonstrates that it is not the size of the testicular Leydig cell population which determines the ability of Leydig cells to produce testosterone *in vitro*. The majority of testes examined contained an equal number of light and dark Leydig cells regardless of total population size. It was not possible to deduce if light or dark Leydig cells had different abilities to produce testosterone. Nor was any difference found in the fold responsiveness to stimulation by hCG of either Leydig cell type. In spite of the considerable individual variations in testosterone production the percentage composition of the testis on a volume basis was remarkably consistent. This suggests that it is a biochemical rather than a physical factor that is important with respect to the *in vitro* variations found. Light and dark Leydig cell cytoplasmic volumes correlated with different testicular factors, though the relevance of this to their role as steroid producers was not clear. Germ cell volume correlated with all other measurements taken, suggesting a pervading influence of germ cells on all aspects of testicular function.

6. Intratesticular control mechanisms in rat and man

The preceding chapters have described how it is possible to isolate Leydig cells from the human testis which are responsive to hCG, and appear to be representative of the normal population of Leydig cells in the human. The aim of studies described in the present chapter was to use isolated human Leydig cells to examine intratesticular control of steroidogenesis in man and to compare methods of local regulation in the rat and human testis.

6.1. Introduction

The most important physiological regulator of testosterone production by Leydig cells is LH (Dufau, 1988), yet many other compounds have been shown to modify rat Leydig cell function either *in vivo* or *in vitro* (for review see Heindel & Treinen, 1989). At the same time, because so little is known about human Leydig cell function (Sharpe, 1990) other species must be used as models for human Leydig cells in studies of endocrine and paracrine control of steroidogenesis. The aims of this study were therefore to investigate how testosterone production may be modulated locally in human Leydig cells, by examining the direct effects of various putative paracrine agents on isolated human Leydig cells, and to compare these findings with the effects of the same agents on testosterone production in rat Leydig cells.

The possible paracrine effects of AVP and LHRH on steroidogenesis in rat and man were compared. Both agents were selected for investigation because their effects in the rat have been well-characterised. Rat Leydig cells have LHRH receptors (Bourne *et al.*, 1980), through which LHRH mediates both stimulatory (short-term, Sharpe & Cooper, 1982) and inhibitory effects (long-term, Sharpe *et al.*, 1983). Rat Sertoli cells in culture produce an LHRH-like activity (Sharpe *et al.*, 1981) detectable in

interstitial fluid. Similarly in the rat, Leydig cells have AVP (V_1) receptors (Meidan & Hsueh, 1985), an AVP-like factor has been localised to the testis (Kasson *et al.*, 1985) and AVP has been shown to have *in vitro* effects on rat Leydig cell testosterone production (Sharpe & Cooper, 1987, Tahri-Joutei & Pointis, 1988).

More recently evidence has accumulated that suggests the involvement of endogenous benzodiazepines in the intracellular regulation of steroidogenesis in the rat. As was the case for AVP and LHRH, peripheral benzodiazepine receptors have been localised to the rat testis (Anhoult *et al.*, 1986), the ligand for these receptors (diazepam binding inhibitor, DBI) has been found in the testis (Rheame *et al.*, 1990), and benzodiazepines such as diazepam, have been shown to have *in vitro* effects on rat Leydig cell testosterone production (Papadopoulos *et al.*, 1990).

The final compound to be investigated as a potential paracrine regulator of steroidogenesis in human Leydig cells, atrial natriuretic peptide (ANP), was included for different reasons. Unlike AVP, LHRH and benzodiazepines, the effect of ANP on isolated rat Leydig cells is unclear. Some groups report a stimulatory effect of ANP on rat Leydig cell cultures (Foresta *et al.*, 1987), and others say that while ANP is able to stimulate testosterone production by isolated mouse Leydig cells, it has no effect on rat Leydig cells (Muhkopadhyay *et al.*, 1986). However, Foresta *et al.* (1991) report that infusion of ANP into the cubital vein of men increases the concentration of plasma testosterone measured in the internal spermatic vein, without affecting the concentration of gonadotrophins. This implies that ANP has a direct effect on human Leydig cells.

The effects of these compounds on basal and hCG-stimulated testosterone production by rat and human Leydig cells were therefore investigated.

6.2. Experimental Procedures

Rat and human Leydig cells were prepared using the methods described in Chapter 3. The effects of various *in vitro* treatments on testosterone production over periods of either 4 and/or 20h were assessed. The effects of the treatments on both basal and hCG-stimulated testosterone production were examined. A concentration of 30IU/ml hCG (Intervet U.K. Ltd, Cambridge) was used to maximally stimulate Leydig cell cultures. The hCG was diluted in culture medium. Preparation of the drug solutions used to treat the cultures is described below.

LHRH-A : A 1.5mg/ml commercial preparation of [D-Ser(tBu)^6,Pro^9-NHEt] LHRH, (buserelin, 'LHRH-A'; Hoescht AG, Frankfurt) was diluted to concentrations between 10^{-8} and 10^{-6}M using culture medium. The stock solution was stored at 4°C.

AVP : 1mg of arginine vasopressin (Sigma) was dissolved in 1ml of culture medium. Aliquots of 15µl were stored frozen for up to 3 months. When required, 10µl of the thawed stock solution were further diluted to give a final concentration of 10^{-6}M.

Diazepam : Diazepam (Sigma) was stored frozen as a stock solution of 10^{-4}M in 1% DMSO (BDH). Thawed aliquots were further diluted in culture medium to concentrations between 10^{-8} and 10^{-6}M.

Rat DBI : The rat specific form of diazepam binding inhibitor (rDBI; Bachem Inc., Essex) was dissolved in double distilled water to give a stock solution of 10^{-4}M. Aliquots were stored frozen for up to 2 months. When required, thawed aliquots were further diluted in culture medium to concentrations between 10^{-8} and 10^{-6}M.

Human DBI : The human specific form of diazepam binding inhibitor (hDBI; Bachem Inc., Essex) was prepared in the same way as rDBI, described above.

ANP : Atrial natriuretic peptide (1-28), human sequence (ANP; Bachem Inc., Essex) was dissolved in double distilled water to give a stock solution of 10^{-4}M. Aliquots were stored frozen until required and were then diluted further in culture medium to concentrations between 10^{-8} and 10^{-6}M.

6.3. Results

LHRH-A : Concentrations of 10^{-6}-10^{-8}M LHRH-A had significant stimulatory effects (p <0.01)on basal testosterone production by rat Leydig cells. The 3 doses had equivalent effects on testosterone production during a 4h culture period (Figure 6.1.A). However, following 20h in culture only 10^{-6}M LHRH-A significantly stimulated testosterone production (p <0.01; Figure 6.2.A). At neither time point did rat Leydig cells respond to LHRH-A in the presence of a maximally-stimulating dose of hCG (Figures 6.1.B and 6.2.B). As there was no difference in the response of rat Leydig cells to the LHRH-A concentration range used, a single dose of 10^{-6}M LHRH-A was used with human Leydig cell cultures. On at least 3 (4h cultures) and 4 (20h cultures) separate occasions human Leydig cells were isolated and incubated with LHRH-A. Due to the marked individual variation in testosterone production by isolated human Leydig cells it was not possible to collate results obtained from different cell preparations. Rather, a representative result is presented as a figure, while results from the remaining experiments are given in an associated table. In 2 out of 3 experiments, LHRH-A had a significant stimulatory effect on basal testosterone synthesis after 4h of culture (p <0.05; Figure 6.1.C and Table 6.1.). After 20h in culture LHRH-A still significantly stimulated basal testosterone production by isolated human Leydig cells in 3 out of 4 experiments (Figure 6.2.C and Table 6.1.). As with rat Leydig cells, human Leydig cells did not respond to LHRH-A in the presence of hCG.

AVP : Basal testosterone production by rat Leydig cells was stimulated by AVP (10^{-6}-10^{-8}M) at 4h (p <0.01; Figure 6.3A), but this stimulatory effect was absent after 20h of culture (Figure 6.4A). Basal testosterone production by human Leydig cells was stimulated significantly by AVP at

4h in 2 out of 3 experiments (p <0.05; Figure 6.3C and Table 6.2). After 20h of culture basal testosterone production by human Leydig cells was enhanced by AVP, although the stimulation only reached significance in 1 out of 3 experiments (Figure 6.4.C and Table 6.2.). At neither time-point did either rat or human Leydig cells stimulated with hCG respond to AVP (Figures 6.3.B and D, 6.4.B and D and Table 6.2.).

Diazepam : Diazepam induced a significant, dose-dependent stimulation of basal testosterone production by rat Leydig cells during a 20h culture period (Figure 6.5.A). When individual experiments were considered a small consistent stimulatory effect of diazepam was seen, which was statistically significant; however, when the results of 5 separate experiments were pooled, this effect was obscured by the differing responses to hCG in each experiment (Figure 6.5.B). In contrast human Leydig cells did not respond to even the highest concentration of diazepam either under basal or hCG-stimulated conditions (Figure 6.5.C and D and Table 6.3.).

Rat DBI : Rat DBI also induced a significant dose-dependent stimulation of basal testosterone production by rat Leydig cells during a 20h culture period (Figure 6.6A), but had no effect on hCG-stimulated testosterone production (Figure 6.6B). Human Leydig cells did not respond to rat DBI under either basal or hCG-stimulated conditions (Figure 6.6 C and D and Table 6.3).

Human DBI : Neither rat nor human Leydig cells responded to human DBI under either basal or hCG-stimulated conditions (Figure 6.7 and Table 6.3).

ANP : There was no response to ANP by either rat or human Leydig cells under basal or hCG-stimulated conditions (Figure 6.8 and Table 6.4).

Figure 6.1. The effects of LHRH-A on testosterone production by rat and human Leydig cells cultured for 4h.

A. basal testosterone production by rat Leydig cells (n=5). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=5). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from basal values.

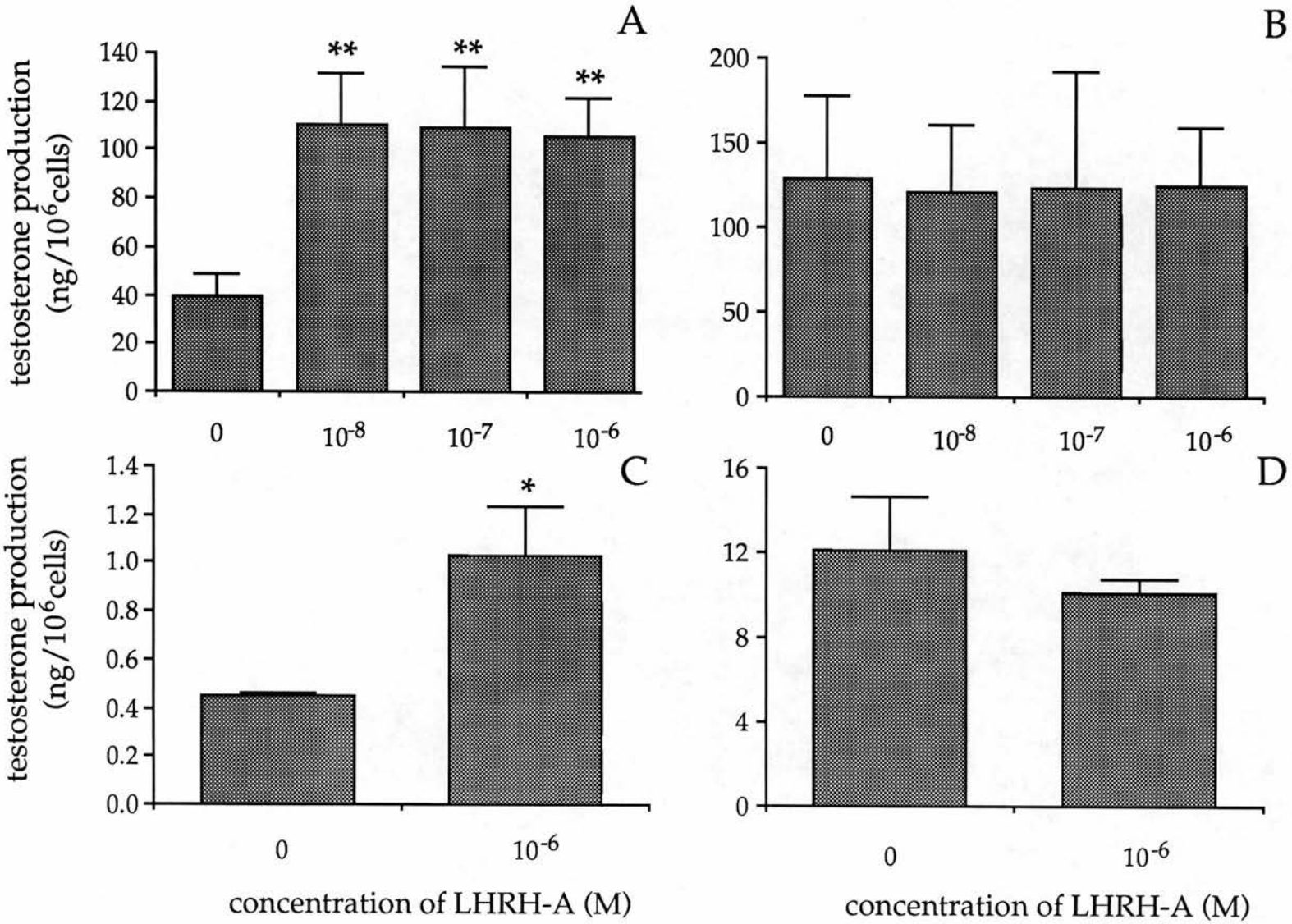

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
**
**
**
*
0
10^-8
10^-7
10^-6
concentration of LHRH-A (M)
concentration of LHRH-A (M)

Figure 6.2. The effects of LHRH-A on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=5). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=5). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from basal values.

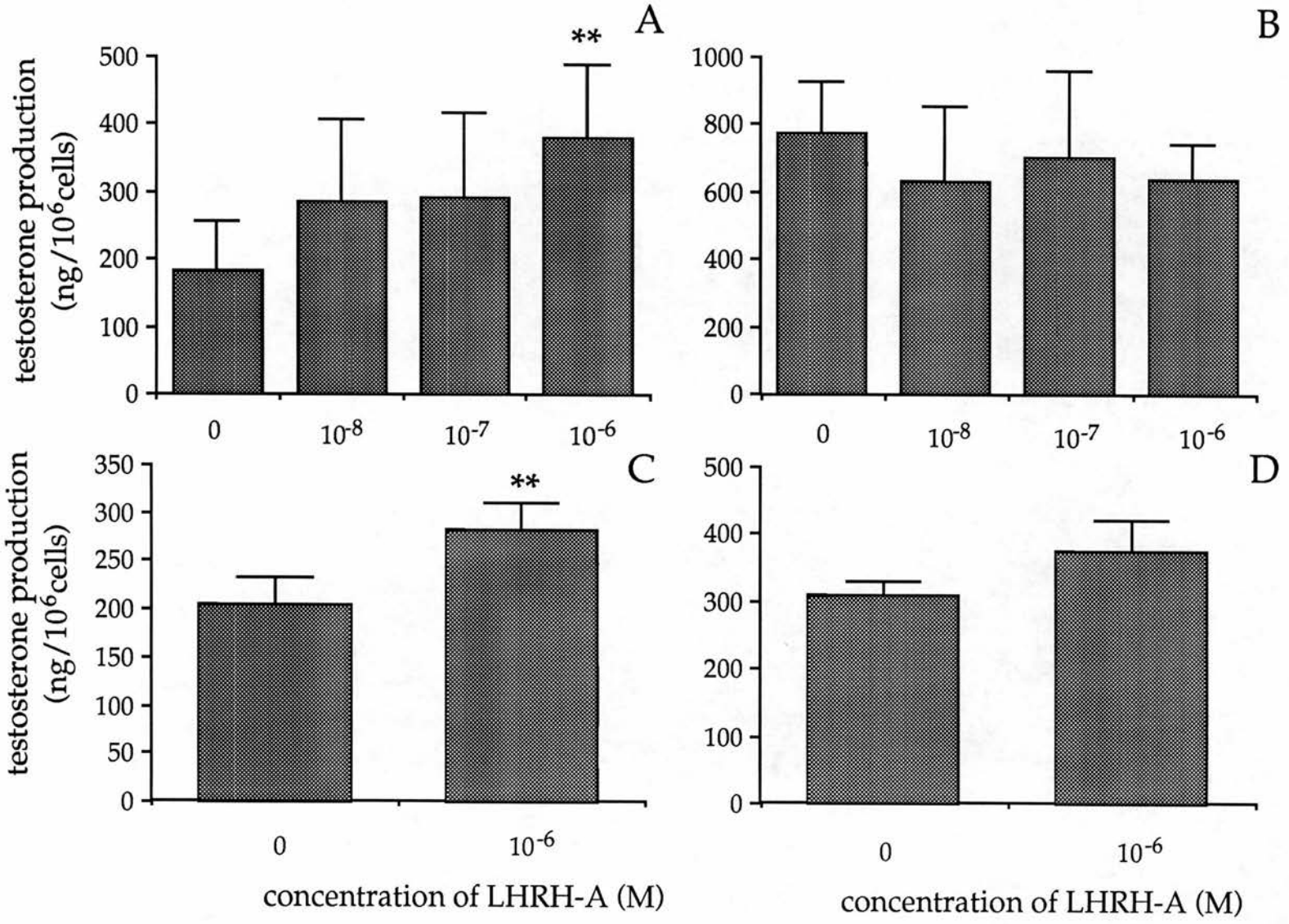

A
B
C
D
**
**
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
500
400
300
200
100
0
1000
800
600
400
200
0
350
300
250
200
150
100
50
0
0
10^-8
10^-7
10^-6
concentration of LHRH-A (M)
concentration of LHRH-A (M)

Table 6.1. The effects of LHRH-A on testosterone production by human Leydig cells cultured for 4 or 20h.

Each value in the table is the mean±S.D. of triplicate wells for further experiments with human Leydig cells. Testosterone was measured as ng/10^6 cells. The results of individual experiments have been presented because of the variability in testosterone production between preparations (see chapter 4). *p <0.05, **p <0.01, significantly different from basal values.

A. 4 hours

basal	10^{-6}M LHRH-A	hCG	hCG +10^{-6}M LHRH-A
0.006±0.004	0.06±0.12*	4.08±1.6	3.56±0.88
1.5±0.14	2.6±0.33	54.2±1.99	43.2±3.96

B. 20 hours

basal	10^{-6}M LHRH-A	hCG	hCG +10^{-6}M LHRH-A
11.4±1.1	17.2±2.1**	16.4±5	18.3±7.3
235.8±36.1	373.99±57.7	1245.1±148.6	1242.4±58.18
150.46±16.9	217.1±22.5*	–	–

Figure 6.3. The effects of AVP on testosterone production by rat and human Leydig cells cultured for 4h.

A. basal testosterone production by rat Leydig cells (n=4). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=4). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from basal values.

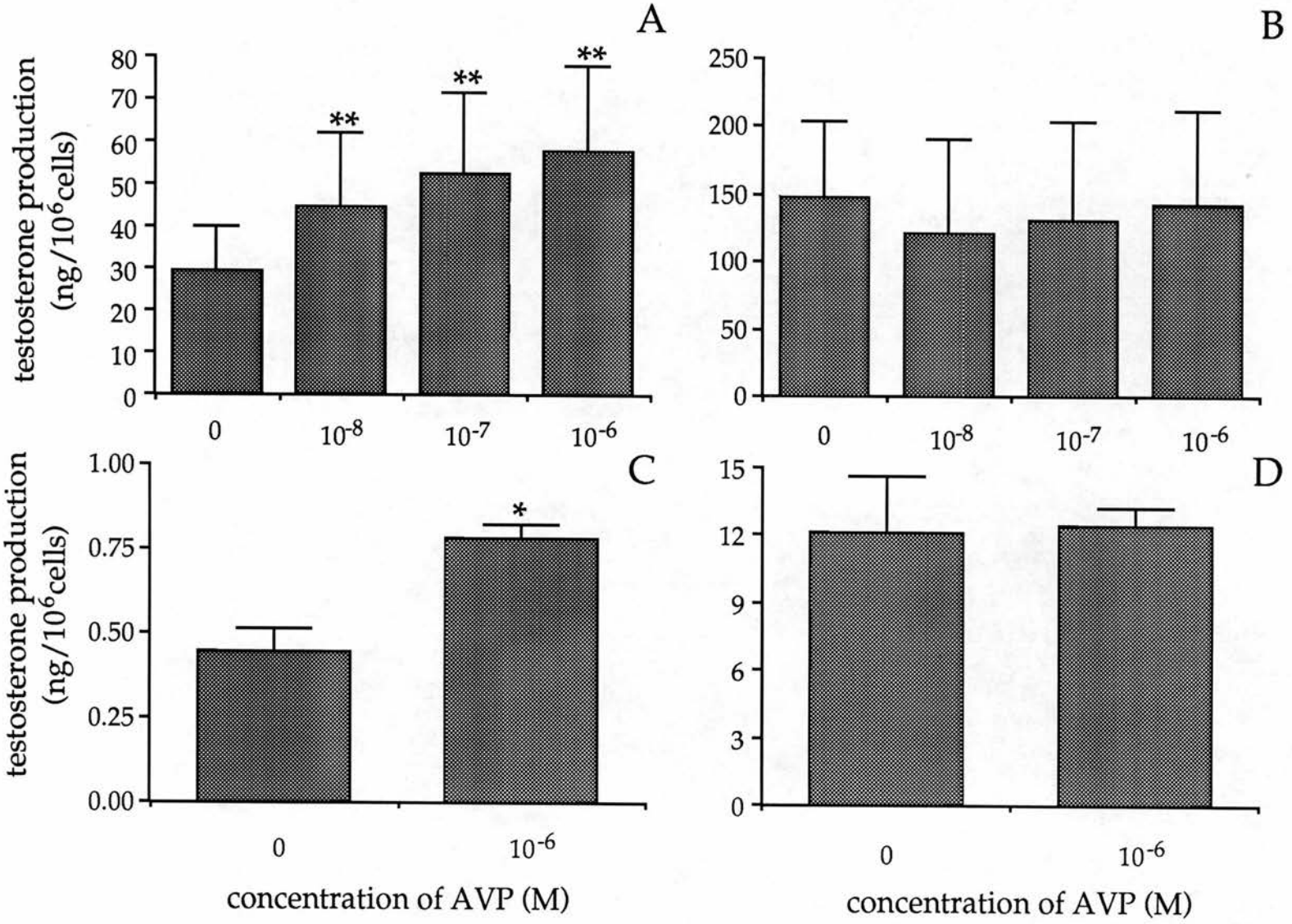

A
B
C
D
testosterone production
(ng/10^6cells)
testosterone production
(ng/10^6cells)
80
70
60
50
40
30
20
10
0
**
**
**
250
200
150
100
50
0
1.00
0.75
0.50
0.25
0.00
*
15
12
9
6
3
0
0
10^-8
10^-7
10^-6
0
10^-6
concentration of AVP (M)
concentration of AVP (M)

Figure 6.4. The effects of AVP on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=5). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=5). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. *p <0.05, **p <0.01, significantly different from basal values.

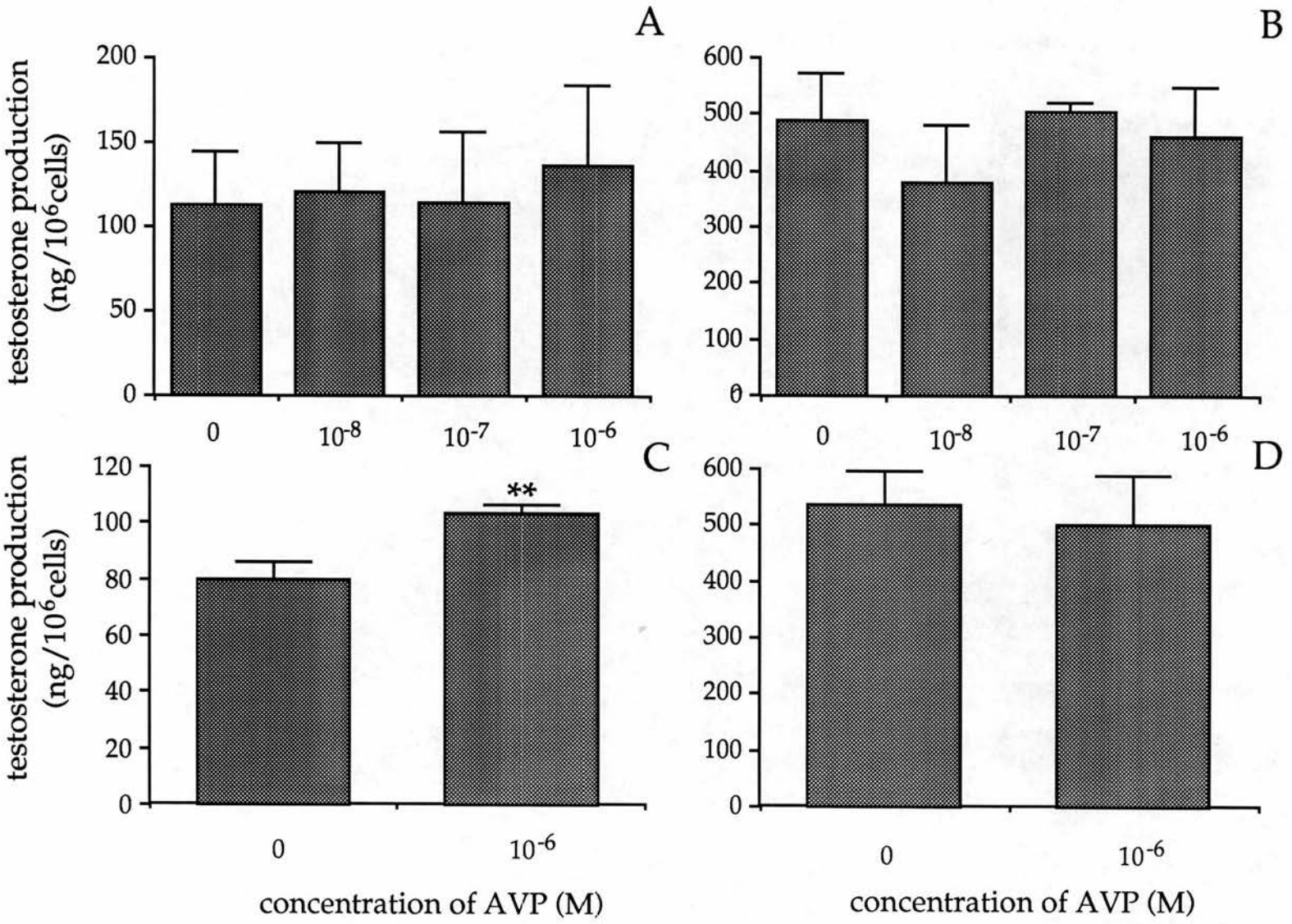

A
B
C
D
testosterone production
(ng/10^6cells)
200
150
100
50
0
600
500
400
300
200
100
120
80
60
40
20
**
0
10^-8
10^-7
10^-6
concentration of AVP (M)

Table 6.2. The effects of AVP on testosterone production by human Leydig cells cultured for 4 or 20h.

Each value in the table is the mean±S.D. of triplicate wells for further experiments with human Leydig cells. Testosterone was measured as ng/10^6 cells. *p <0.05, significantly different from basal values.

A. 4 hours

basal	10^{-6}M AVP	hCG	hCG +10^{-6}M AVP
0.006±0.004	0.04±0.15*	4.08±1.6	5.3±1.8
1.5±0.14	2.77±0.52	54.2±1.99	54.05±4.43

B. 20 hours

basal	10^{-6}M AVP	hCG	hCG +10^{-6}M AVP
3.62±0.9	3.6±0.49	26.58±5.27	29.67±4.98
1.51±0.44	1.57±0.14	33.9±7.96	36.2±8.29

Figure 6.5. The effects of Diazepam on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=5). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=5). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from basal values.

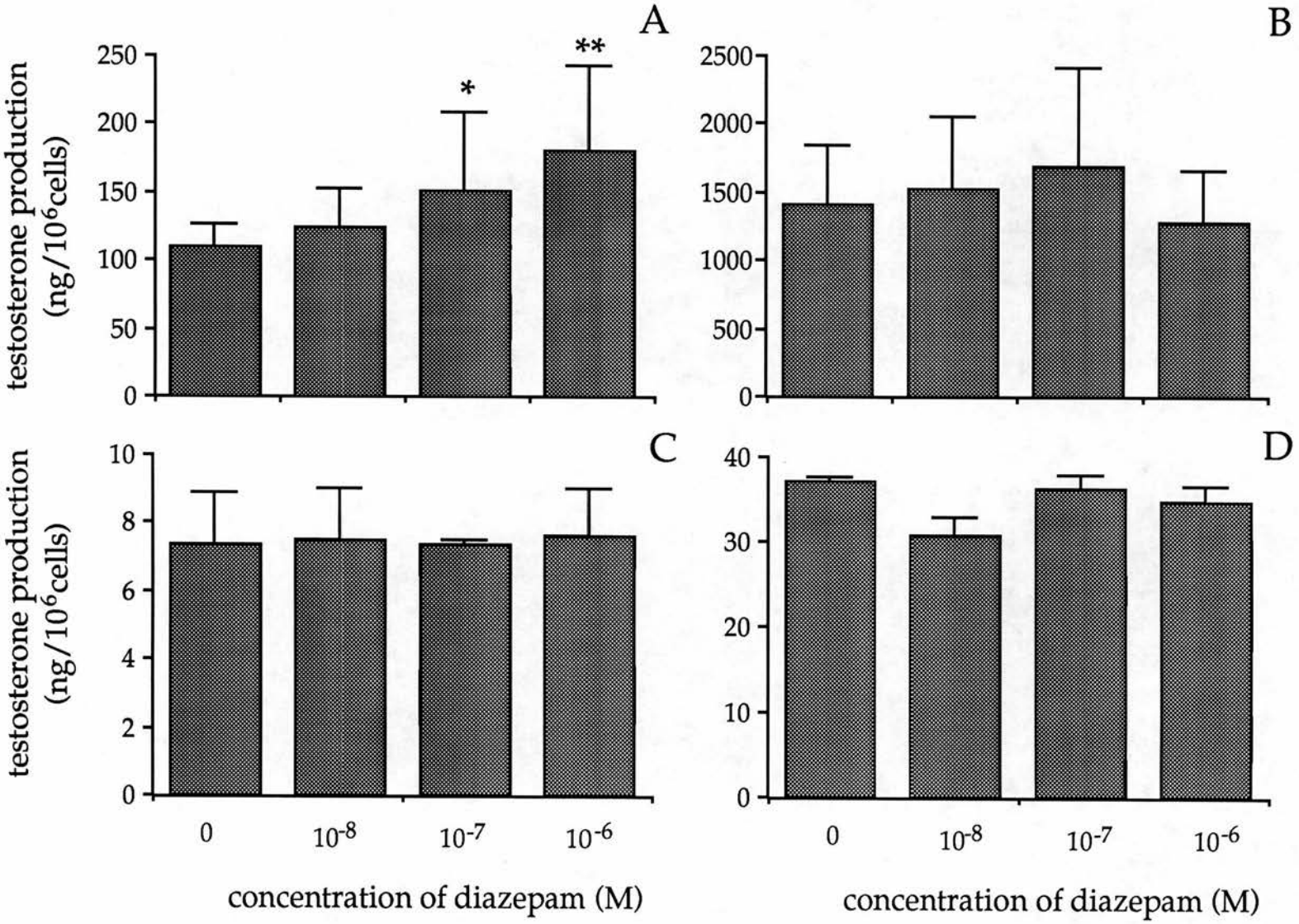

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
250
200
150
100
50
0
2500
2000
1500
1000
500
0
10
8
6
4
2
0
40
30
20
10
0
*
**
0
10^-8
10^-7
10^-6
concentration of diazepam (M)
concentration of diazepam (M)

Figure 6.6. The effects of rat DBI on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=4). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=4). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from basal values.

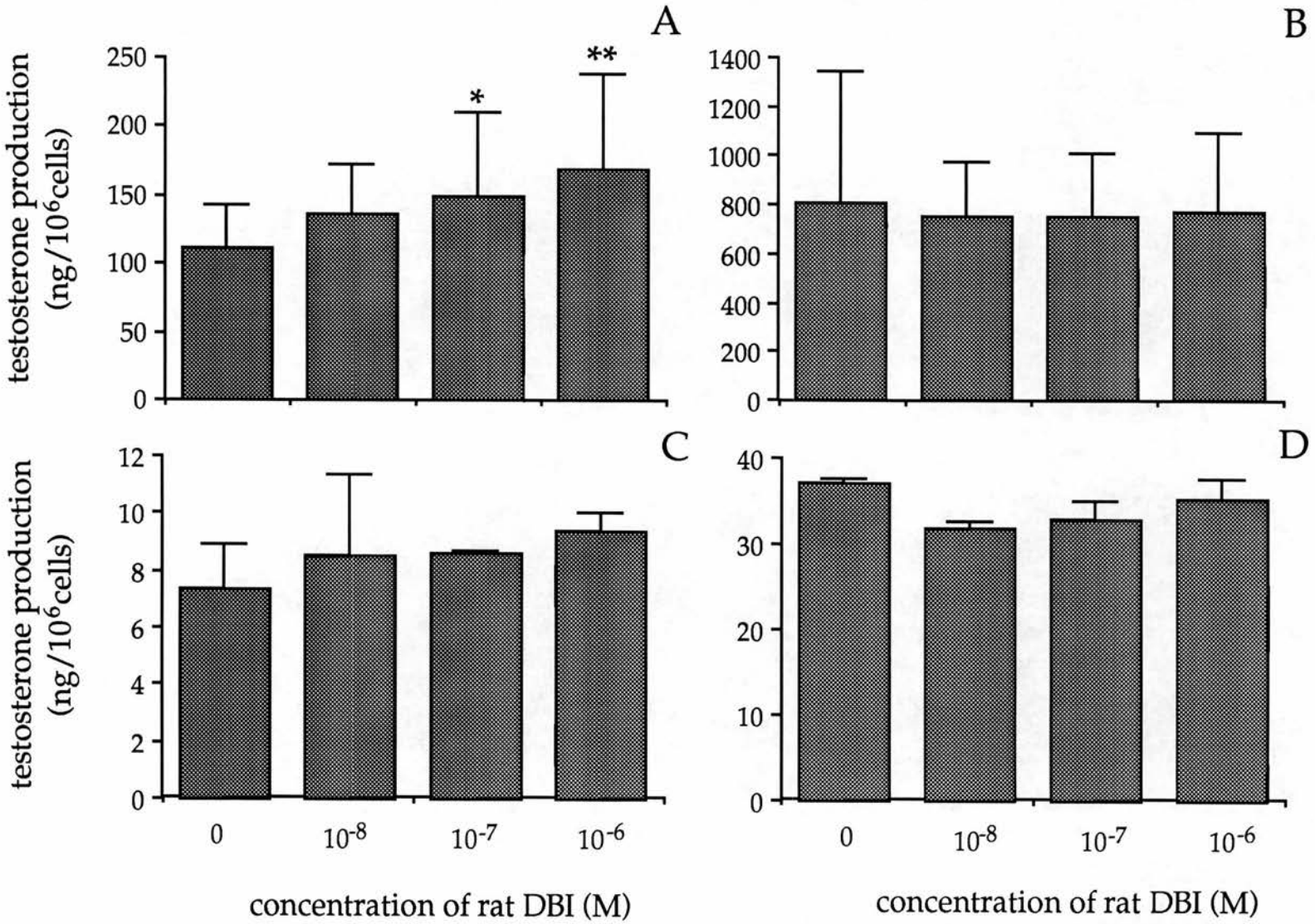

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
250
200
150
100
50
0
*
**
1400
1200
1000
800
600
400
200
0
12
10
8
6
4
2
0
40
30
20
10
0
0
10-8
10-7
10-6
concentration of rat DBI (M)
concentration of rat DBI (M)

Figure 6.7. The effects of human DBI on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=3). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=3). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells.

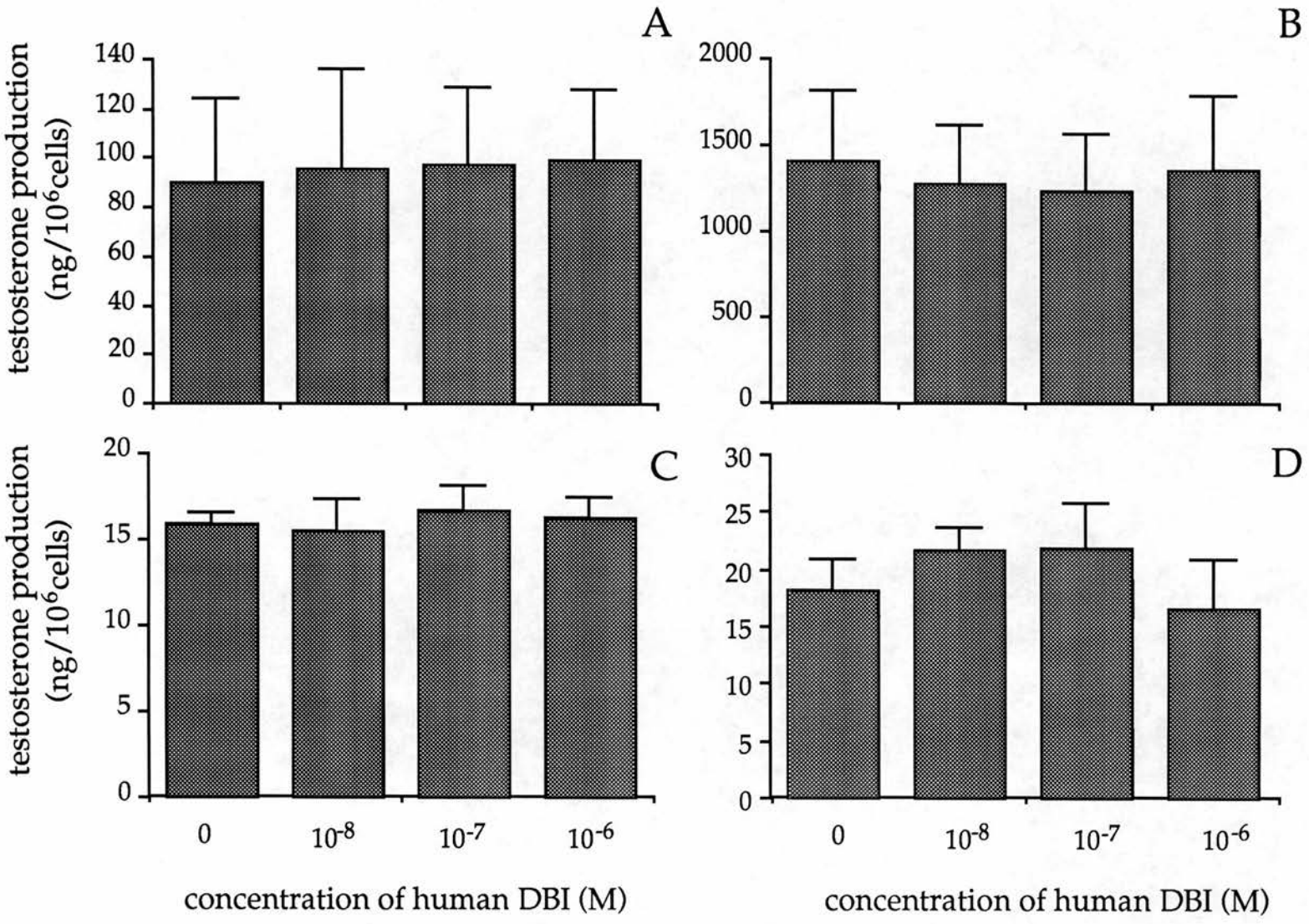

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
140
120
100
80
60
40
20
0
2000
1500
1000
500
0
20
15
10
5
0
30
25
20
15
10
5
0
0
10-8
10-7
10-6
0
10-8
10-7
10-6
concentration of human DBI (M)
concentration of human DBI (M)

Table 6.3. The effects of Diazepam, rat DBI and human DBI on testosterone production by human Leydig cells cultured for 20h.

Each value in the table is the mean±S.D. of triplicate wells for further experiments with human Leydig cells. Testosterone was measured as ng/10^6 cells.

A. Diazepam

basal	10^{-8}M	10^{-7}M	10^{-6}M	hCG	hCG+ 10^{-8}M	hCG+ 10^{-7}M	hCG+ 10^{-6}M
7.37±1.52	7.52±1.47	7.39±0.1	7.62±1.52	37.08±0.49	30.69±2.14	36.38±1.52	34.67±2.02
64.49±3.43	_	63.37±3.43	63.98±3.45	_	_	_	_

B. rat DBI

basal	10^{-8}M	10^{-7}M	10^{-6}M	hCG	hCG+ 10^{-8}M	hCG+ 10^{-7}M	hCG+ 10^{-6}M
53.19±4.18	_	50±23.4	78.17±5.3	_	_	_	_
64.49±3.43	_	63.37±3.43	63.98±3.45	_	_	_	_

C. human DBI

basal	10^{-8}M	10^{-7}M	10^{-6}M	hCG	hCG+ 10^{-8}M	hCG+ 10^{-7}M	hCG+ 10^{-6}M
0.27±0.05	0.267±0.049	0.266±0.05	0.204±0.05	6.59±1.28	_	6.52±1.67	6.59±1.28
0.51±0.01	_	_	0.37±0.03	_	_	_	_

Figure 6.8. The effects of hANP(1-28) on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=3). **B.** hCG-stimulated testosterone production by rat Leydig cells(n=3). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells.

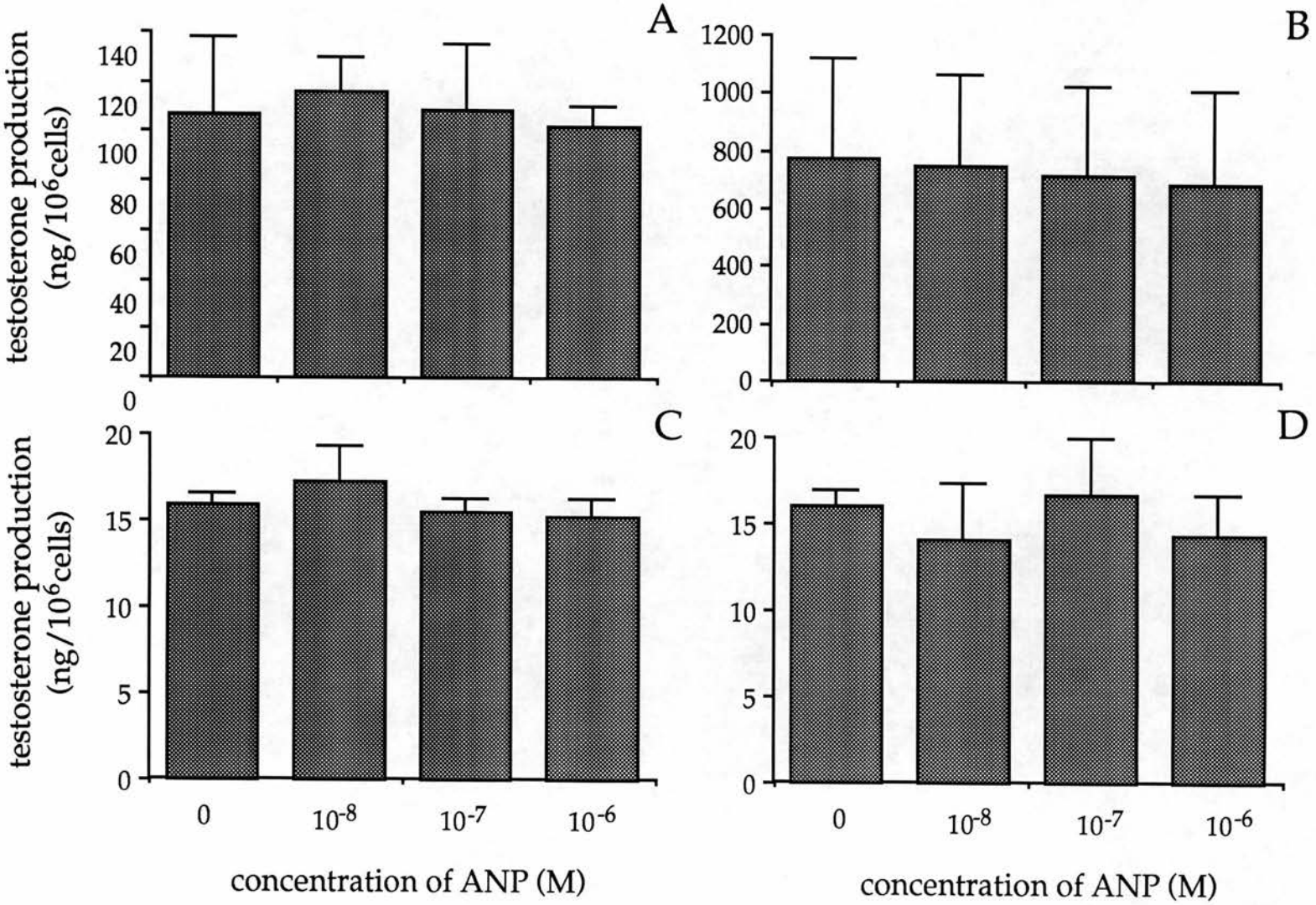

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
140
120
100
80
60
40
20
0
1200
1000
800
600
400
200
0
20
15
10
5
0
0
10^-8
10^-7
10^-6
concentration of ANP (M)
concentration of ANP (M)

Table 6.4. The effects of hANP(1-28) on testosterone production by human Leydig cells cultured for 20h.

Each value in the table is the mean±S.D. of triplicate wells for further experiments with human Leydig cells. Testosterone was measured as ng/10^6 cells.

basal	10^{-6}M ANP	hCG	hCG +10^{-6}M ANP
0.513±0.102	0.605±1.54	1.77±1.07	1.8±1.1

6.4. Discussion

The experiments described in this chapter comprise an investigation into the actions of possible local modulators of steroidogenesis *in vivo*, on testosterone production by isolated human Leydig cells *in vitro*. A comparison was made between the effects of these potential paracrine regulators on human and rat Leydig cells.

The effects of both LHRH-A and AVP are well-documented in the rat. In short-term cultures (<24h) LHRH-A has a stimulatory effect on testosterone production by rat Leydig cells (Sharpe & Cooper, 1987, Cooke & Sullivan, 1985). While such effects in the rat were confirmed in this study, a novel finding was that 10^{-6}M LHRH-A had a direct stimulatory effect on basal, though not hCG-stimulated, testosterone production by human Leydig cells. The effect was seen after both 4 and 20h of culture and closely parallels the response of rat Leydig cells *in vitro* to LHRH-A. Previous studies have not supported a paracrine role for LHRH in the human testis, partly based on the apparent lack of a direct testicular effect of LHRH-A *in vivo* (Rajfer *et al.*, 1987, Vicari *et al.*, 1986). However, these studies examined the ability of LHRH-A to alter hCG-stimulated testosterone production, yet the results described in this chapter for human and rat Leydig cells, and by Sharpe & Cooper (1982; 1987) for rat Leydig cells, demonstrate that the direct effect of LHRH-A on isolated Leydig cells is on basal and not hCG-stimulated testosterone production. The strongest evidence against a paracrine role for LHRH in the human testis is the study by Clayton & Huhtaniemi (1982), which found that while testicular binding sites for LHRH-A were easily demonstrable in the rat, there were no high affinity binding sites for LHRH-A in either human testis or corpus luteum (CL). However, a later study by Popkin *et al.* (1985), detected low affinity binding sites for LHRH-A in both human CL and testis. These low affinity binding sites are now well-described in the human ovary and placenta where they have been shown to mediate biological effects of LHRH and its agonists (Bramley, 1989). These low affinity LHRH binding sites exhibit comparable affinity for LHRH agonists and native LHRH. In contrast, in rat gonads LHRH agonists have 100-1000 times greater affinity for the binding site than LHRH itself. This suggests the existence of a human binding site for LHRH with a different

conformation to its rat counterpart. It is therefore possible that Clayton & Huhtaniemi (1982) were unable to detect LHRH binding sites in extra-pituitary binding sites because they were using an inappropriate agonist for the receptor. It is also possible that human Leydig cells contain fewer LHRH binding sites than do rat Leydig cells. This is true in the case of the LH receptor, where rat Leydig cells are estimated to have 23,500 binding sites/cell, and the human Leydig cell only 1,630 sites/cell (Huhtaneimi *et al.*, 1982). The present finding of stimulatory effects of LHRH-A on basal testosterone production by 3 separate preparations of human Leydig cells is consistent with the presence of low affinity LHRH binding sites in the human testis and also with the description of the biological activity of similar receptors in the human ovary and placenta. However, the nature and physiological role of testicular LHRH is obscure (Sharpe, 1990) and the functional relevance of any direct effect of LHRH in the testis is not clear.

This study has confirmed that the peptide hormone, AVP, has short-term stimulatory effects on basal testosterone secretion by rat Leydig cells (Sharpe & Cooper, 1987, Tahri-Joutei & Pointis, 1988). In addition it has shown that isolated human Leydig cells may also respond to AVP: a significant stimulation of basal testosterone production was seen at 4h in 2 out of 3 separate preparations of human Leydig cells and in 1 out of 3 separate preparations at 20h. AVP did not affect hCG-stimulated testosterone production by either rat or human Leydig cells at 4 or 20h of culture. The absence of a response to AVP in the presence of a supramaximal dose of hCG has been described previously, in purified rat Leydig cell cultures (Sharpe & Cooper, 1987), and in purified mouse Leydig cell cultures (Tahri-Joutei & Pointis, 1988). In the rat there is strong evidence for a potential paracrine role for testicular AVP. Kasson *et al.* (1985), established the presence of AVP-like peptides in the rat testis, where they may have local effects on steroidogenesis (Tahri-Joutei & Pointis, 1988) which are mediated through specific (V_1) receptors localised to Leydig cells (Meidan & Hsueh, 1985). Kasson *et al.* (1985) demonstrated the presence of an immunoreactive AVP-like substance in the rat testis. The apparent testicular content of AVP was 600pg/testis, indicating the presence of AVP in sufficient amounts to exert a local modulatory effect on testicular function.

While no study has looked for the presence of AVP receptors on human Leydig cells, an immunoactive vasopressin-like material has been isolated from human testicular tissue (Nicholson *et al.*, 1984). This, together with the results of this study showing direct stimulatory effects of AVP on testosterone production by human Leydig cells *in vitro*, supports a role for AVP as a potential paracrine regulator of human steroidogenesis. However the physiological significance of these observations is unclear, as Tahri-Joutei & Pointis (1989) have shown that an intratesticular injection of AVP does not modify testosterone levels when measured 5-6 hours later in the mouse testis, neither does such an injection alter the amounts of testosterone measurable in rat testicular interstitial fluid (Sharpe & Cooper, 1987).

The evidence that endogenous benzodiazepines may have a role in the local control of steroidogenesis is reasonably strong. There are two classes of benzodiazepine receptor, one class is entirely localised within the CNS, while the second class of 'peripheral' benzodiazepine receptors (PBR) is found in high levels in some steroidogenic organs, including the testis and the adrenal cortex (De Souza *et al.*, 1980). It is now known that PBR are located primarily in mitochondrial membranes (Antkiewicz-Michaluk *et al.*, 1988) and the cell type that contains the majority of PBR is the Leydig cell (Calvo *et al.*, 1990). Anhoult *et al.* (1986) showed that the density of rat adrenal and testicular PBR decreases after hypophysectomy. The demonstration that PBR density could be altered by hormone manipulation suggested a possible role for endogenous benzodiazepines in steroidogenesis. It has since been shown that PBR agonists, such as Ro 5-4864 or diazepam, have stimulatory effects on testosterone production by decapsulated rat testes (Ritta *et al.*, 1987), crude interstitial cell suspensions (Ritta & Calandra, 1989), purified rat Leydig cells and also in MA-10 cells (Papadopoulos *et al.*, 1990). The endogenous ligand for PBR is believed to be a polypeptide termed diazepam binding inhibitor (DBI) because of its ability to displace diazepam from PBR. DBI has been isolated and characterised from rat brain (Guidotti *et al.*, 1983), and has since been localised to the rat testis, specifically to Leydig cells (Rheaume *et al.*, 1990). In agreement with the study by Papadopoulos *et al.* (1990), this study also demonstrated that diazepam causes dose-dependent stimulation of basal testosterone production by rat Leydig cells. However, isolated human

Leydig cells showed no response to diazepam even at the highest concentration tested. Diazepam is not a specific agonist for PBR but has affinity for both central and peripheral benzodiazepine receptors. DBI, which occurs in both human and rat forms is the preferred ligand for the PBR. The effects of both forms of DBI on testosterone production by rat and human Leydig cells were examined. Basal testosterone secretion by rat Leydig cells was stimulated by rat DBI as has been shown previously (Papadopoulos *et al.*, 1991), but the human form of DBI had no effect on testosterone production by rat Leydig cells. Testosterone secretion by human Leydig cells was not affected by either the rat or human form of DBI. Kreuger & Papadopoulos (1990) have shown that modulation of steroid production by ligands of PBR involves activation of intramitochondrial cholesterol transport. As the transfer of cholesterol from intracellular stores to the outer mitochondrial membrane and then to the inner mitochondrial membrane is the rate limiting step in steroidogenesis, it is possible that one of the most crucial points in the steroidogenic pathway may involve different control mechanisms in rat and human Leydig cells. However, rat DBI can be digested to smaller peptide fragments such as ODN (octadecaneuropeptide) which can also displace diazepam from PBR. It may be that in the human testis it is a peptide fragment of DBI which is active and isolated human Leydig cells lack the ability to form this fragment *in vitro*. However, given the ability of both diazepam and rat DBI to stimulate testosterone production in rat Leydig cells and the inability of both diazepam and human DBI to affect testosterone production by human Leydig cells, there is a strong possibility that a genuine difference occurs in the mode of intramitochondrial cholesterol transport in the rat and human testis.

ANP has also been proposed as having a role in the local regulation of testosterone production. It has been shown to stimulate testosterone and cGMP production in isolated mouse Leydig cells (Bex & Corbin, 1985, Mukhopadhyay *et al.*, 1986). Foresta & Mioni (1988) showed that hANP (1-28) also had a stimulatory effect on testosterone and cGMP production by rat Leydig cells; however whilst Mukhopadhyay *et al.* (1986) were able to show an increase in cGMP by isolated rat Leydig cells, they found no effect of ANP on testosterone production. Foresta *et al.* (1991) demonstrated that an infusion of 100μg/ml ANP into the cubital vein

had a significant effect on plasma testosterone measured in blood sampled from the internal spermatic vein in men undergoing varicocele embolization. An increase in testosterone concentration in the internal spermatic vein was evident within 15 minutes of the ANP infusion, while no significant rise in testosterone concentration was measurable in peripheral plasma. There was no effect on serum gonadotrophin levels in blood sampled from either the internal spermatic or peripheral veins. The authors suggested that ANP exerts a stimulatory effect on testicular steroidogenesis in men without modifying pituitary gonadotrophin secretion, i.e. they propose that ANP has a direct effect on human Leydig cells. The results of experiments presented in this chapter are consistent with the findings of Mukhopadhyay *et al.* (1986) in that no effect of ANP on testosterone production by rat Leydig cells was demonstrable. Neither were human Leydig cells found to respond to ANP. When considering the results obtained by Foresta *et al.* (1991), the lack of a direct effect of ANP on isolated human Leydig cells, could indicate that the responsiveness of human Leydig cells to ANP is lost *in vitro*, possibly as a consequence of the isolation procedure. However, it is also possible that the changes in spermatic vein testosterone levels measured by Foresta *et al.* (1991) reflect changes in the rate of testicular blood flow caused by the vasodilatory effect of ANP. Until this can be clarified there does not seem to be any evidence for a paracrine role for ANP in the human testis.

In summary, the work described in this chapter examines how steroidogenesis may be altered at the intratesticular level in rat and human Leydig cells. In many respects modulation of rat and human Leydig cell function appeared to be very similar; both rat and human Leydig cells were stimulated to produce testosterone *in vitro* by AVP and an LHRH-A, and neither showed an *in vitro* response to ANP. However, differences were also discovered, as unlike rat cells, isolated human Leydig cells did not respond to benzodiazepines. As rat Leydig cells are often used as a model of what might happen in man, it is important to know that human Leydig cells do not show responses that are markedly different to those shown by rat Leydig cells. The results of the present study suggest that, overall, rat Leydig cells are a good model for human Leydig cells. Studies described later in this thesis on protein secretion (chapter 8) and toxicology (chapter 10) also support this conclusion.

7. Indirect modulation of Leydig cell function by germ cells

The previous chapter has demonstrated how isolated Leydig cells can be used to investigate the effects of putative paracrine agents. However while it is possible to demonstrate effects *in vitro* it remains uncertain whether such results can be extrapolated to the *in vivo* situation. Cultured rat Sertoli cells produce factors(s) which are able to stimulate Leydig cell steroidogenesis *in vitro*, and it has been demonstrated that the production of such factors can be modulated by germ cells. This raises the possibility that in vivo there is a cascade of cell-cell interactions with the presence/absence of particular germ cells leading to alterations in the Leydig cells, presumably via the mediation of Sertoli-cell secreted factors. The present chapter describes studies which were designed to test this possibility.

7.1. Introduction

The ability of isolated rat Leydig cells to produce testosterone *in vitro* can be affected by the addition of a large number of compounds (for review see Heindel & Treinen, 1989 and the previous chapter). While there is only limited evidence supporting the production and function of such compounds *in vivo*, there is abundant evidence of regulatory interactions between testicular cell types which reach across the two compartments of the testis. Such local control systems would allow the functional responses of cells to be more finely controlled than would be possible through pituitary regulation alone.

Several studies have shown that damage to the seminiferous epithelium, caused by local administration of anti-androgens (Aoki & Fawcett, 1978), γ-irradiation (Papadopoulos *et al.*, 1987b), cryptorchidism (Risbridger *et al.*, 1981) or local testicular heating (Jegou *et al.*, 1984), can secondarily affect Leydig cell structure and/or function. More direct evidence of a functional interaction between germ cells, Sertoli cells and

Leydig cells was provided by Onoda *et al.* (1991), who showed that Sertoli cells in culture secrete a protein(s) which stimulates Leydig cell steroidogenesis, and that pachytene spermatocyte (PS) proteins were able to modulate the polarised secretion of the Sertoli cell protein(s). While this is the first direct evidence of germ cell-Sertoli cell-Leydig cell interactions, it still has several limitations. These include the use of immature Sertoli cells and the separation and isolation of cell types with consequent changes in their function. An alternative approach involving the use of the germ cell specific toxin, methoxyacetic acid (MAA) was outlined by Bartlett *et al.* (1988). Administration of MAA to rats causes selective and stage-specific destruction of PS. A dose of 650 mg/kg results in PS degeneration at all stages of the spermatogenic cycle except for PS at early-mid stage VII (Foster *et al.*, 1983, Creasy *et al.*, 1985). At this dose MAA has no other discernable adverse effects on the rats, and the unaffected germ cells continue to progress normally through spermatogenesis (Ratnasooriya & Sharpe, 1989). The mechanism by which MAA exerts this selective effect is unknown, neither is it understood how a subset of PS at early-mid stage VII are resistant to the action of MAA. However, Chowdhury & Steinberger (1964) found that local heating of the testis also only affected PS at specific stages.

The consequences of maturation depletion allow MAA to be used to generate testes that are depleted of specific types of germ cells, while all other testicular cells are intact, leaving the majority of cell-cell interactions to proceed as normal. Thus at 3 days after administration of MAA seminiferous tubules at all stages of the spermatogenic cycle except VIII-XI are depleted of PS. By 7 days, seminiferous tubules at stages VII-XIII have been depleted of PS, while seminiferous tubules at stages II-VI are depleted of round spermatids (RS) because of maturation depletion. At 14 days post-MAA treatment, all tubules apart from those at stage VIII have no RS, while by day 21 seminiferous tubules at stages IV-VIII and IX-XI lack elongating and elongate spermatids (ES).

The experiments described in this chapter have used MAA to deplete the testes of specific types of germ cells in order to investigate the effect of selective germ cell depletion *in vivo* on the function of Leydig cells isolated from these testes and cultured *in vitro*. The effects of such

interactions between testicular cells on the *in vitro* actions of a putative paracrine regulator of Leydig cell function (LHRH) were also examined.

7.2. Experimental Procedures

7.2.1. Preparation of Methoxyacetic Acid (MAA)

To prepare a stock solution of MAA (Aldrich) suitable for oral administration, 10.8ml MAA (pH 0.5) were diluted with concentrated sodium hydroxide to between pH 7.0-7.4. The solution was made up to a final volume of 45ml with saline. This solution was prepared when required.

7.2.2. Dosing regimen

Adult male rats aged 70-100 days were administered a single oral dose of 650mg/kg MAA by gavage. Control animals received an equivalent volume of saline alone. This dose of MAA has been shown previously to deplete rat seminiferous tubules of the majority of pachytene and later spermatocytes within about 24 hours of administration (Bartlett *et al.*, 1988).

7.2.3. Isolation of Leydig cells from germ cell depleted testes

At predetermined time points, namely, 3, 7, 14, 21 and 42 days post-treatment, Leydig cells from 4 control and 4 treated rats were isolated in parallel using the methods described in Chapter 3. Leydig cells from each group were incubated for 20h. Basal and maximal hCG-stimulated testosterone production were studied. In addition the response of the Leydig cells to the LHRH agonist, buserelin (LHRH-A) was assessed.

7.3. Results

The basal testosterone output of Leydig cells isolated from control and MAA-treated rats at each time-point, is shown in Figure 7.1. Leydig cells isolated from testes in which the major germ cell types depleted were a combination of RS and PS (MAA+7 days), or ES (MAA+21 days) did not differ from Leydig cells isolated from control testes in their ability to produce testosterone *in vitro*, in the absence of hCG. Leydig cells isolated 3 days after MAA administration produced significantly more testosterone *in vitro* (p <0.01) than did Leydig cells isolated from control rat testes. A similar increase (p <0.05) in Leydig cell responsiveness was observed at 14 days after MAA-treatment. At 3 days after MAA-treatment testes are depleted of 80-100% of PS and later spermatocytes (Ratnasooriya & Sharpe, 1989), whereas by 14 days after MAA administration seminiferous tubules are depleted mainly of RS. The effect of *in vivo* administration of MAA on the ability of isolated Leydig cells to respond to hCG stimulation *in vitro* is shown in Figure 7.2. The production of testosterone in response to hCG showed marked between-experiment variation in both control and MAA-treated groups and overall there was no evidence for any significant change as a result of the *in vivo* treatment (ANOVA; p = 0.688). At 42 days after MAA treatment, by which time a normal full complement of germ cells has been restored (Bartlett *et al.*, 1988), there was no difference in basal testosterone production *in vitro* by Leydig cells isolated from either control or treated rats (Figure 7.1.1).

The effect of the addition of 10^{-6}M LHRH-A to cultures of Leydig cells isolated from control and MAA-treated rats, on days 3, 7, 14 and 21 after administration of MAA are shown in Figure 7.3. LHRH-A had a stimulatory effect on basal testosterone production by isolated Leydig cells in all instances. With Leydig cells isolated from control rats this effect reached significance on days 3 and 14 post-treatment. Figure 7.3A shows the response to LHRH-A of Leydig cells isolated from control and from treated animals 3 days (d3) after administration of MAA. Leydig cells from the control animals show a significant stimulatory response to LHRH-A (p <0.01). The response of Leydig cells from d3 MAA-treated animals to LHRH-A was also significant, but to a lesser degree (p <0.05). In fact, with Leydig cells from control animals the fold increase in testosterone

production caused by LHRH-A was x4.9, while in Leydig cells isolated from d3 MAA-treated animals the fold response fell to x1.46. A similar picture was evident when comparing Leydig cells from control animals with Leydig cells isolated from MAA-treated animals at 14 days (d14) post-treatment (Figure7.3C). Leydig cells from control animals showed a significant stimulatory response (x2.18-fold) to LHRH-A (p <0.05), whilst Leydig cells from d14 MAA-treated animals did not show a statistically significant response to LHRH-A (x1.25-fold). At both 3 and 14 days after MAA treatment, the decline in fold responsiveness to LHRH-A was due to the increase in basal testosterone production (Figure 7.1.) rather than to a decrease in the absolute amount of testosterone produced in response to LHRH-A. When Leydig cells were isolated from MAA-treated rats at 7 and 21 days post-treatment, there was no difference in either basal testosterone production or in testosterone production in response to LHRH-A, between Leydig cells isolated from either treated or control animals.

Figure 7.1. The effects of administration of MAA on basal testosterone secretion by rat Leydig cells at successive intervals after treatment.

The graph shows basal testosterone secretion in control (o) and treated (•) rats. Each point is the mean±S.D of 3 separate experiments. *p <0.05, **p <0.01, treated value significantly different from control value at that time point.

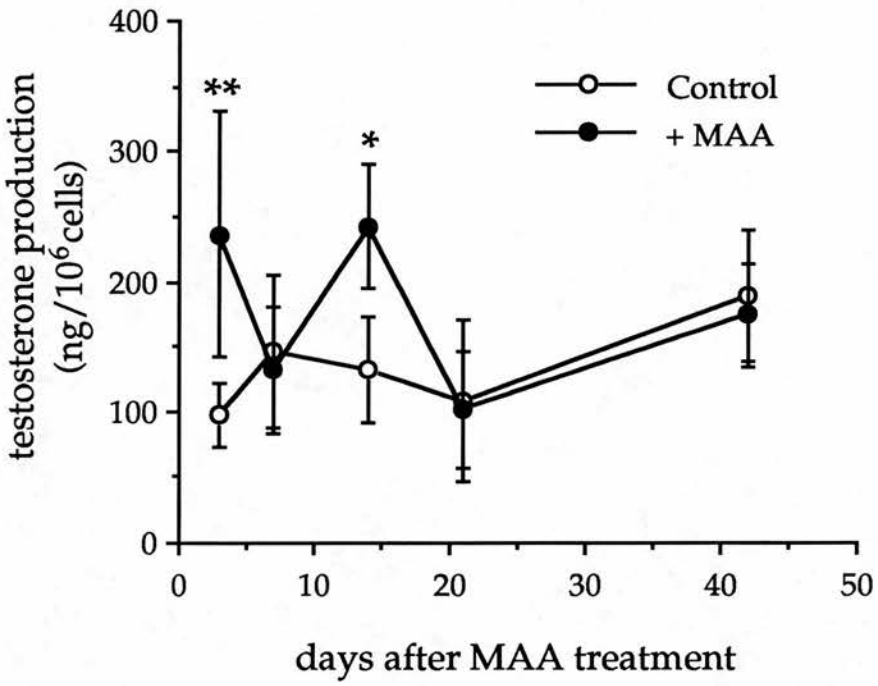

400
300
200
100
0
0
10
20
30
40
50
**
*
Control
+ MAA
testosterone production
(ng/10^6cells)
days after MAA treatment

Figure 7.2. The effects of administration of MAA on hCG-stimulated testosterone secretion by rat Leydig cells at successive intervals after treatment.

The graph shows hCG-stimulated testosterone secretion in control (o) and treated (•) rats. Each point is the mean±S.D of 3 separate experiments.

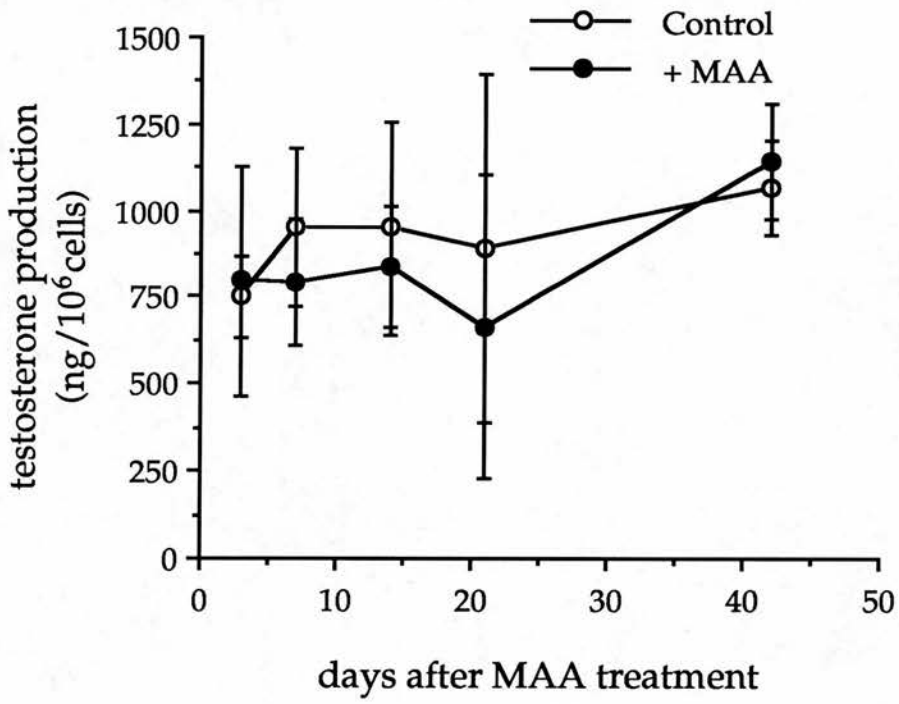
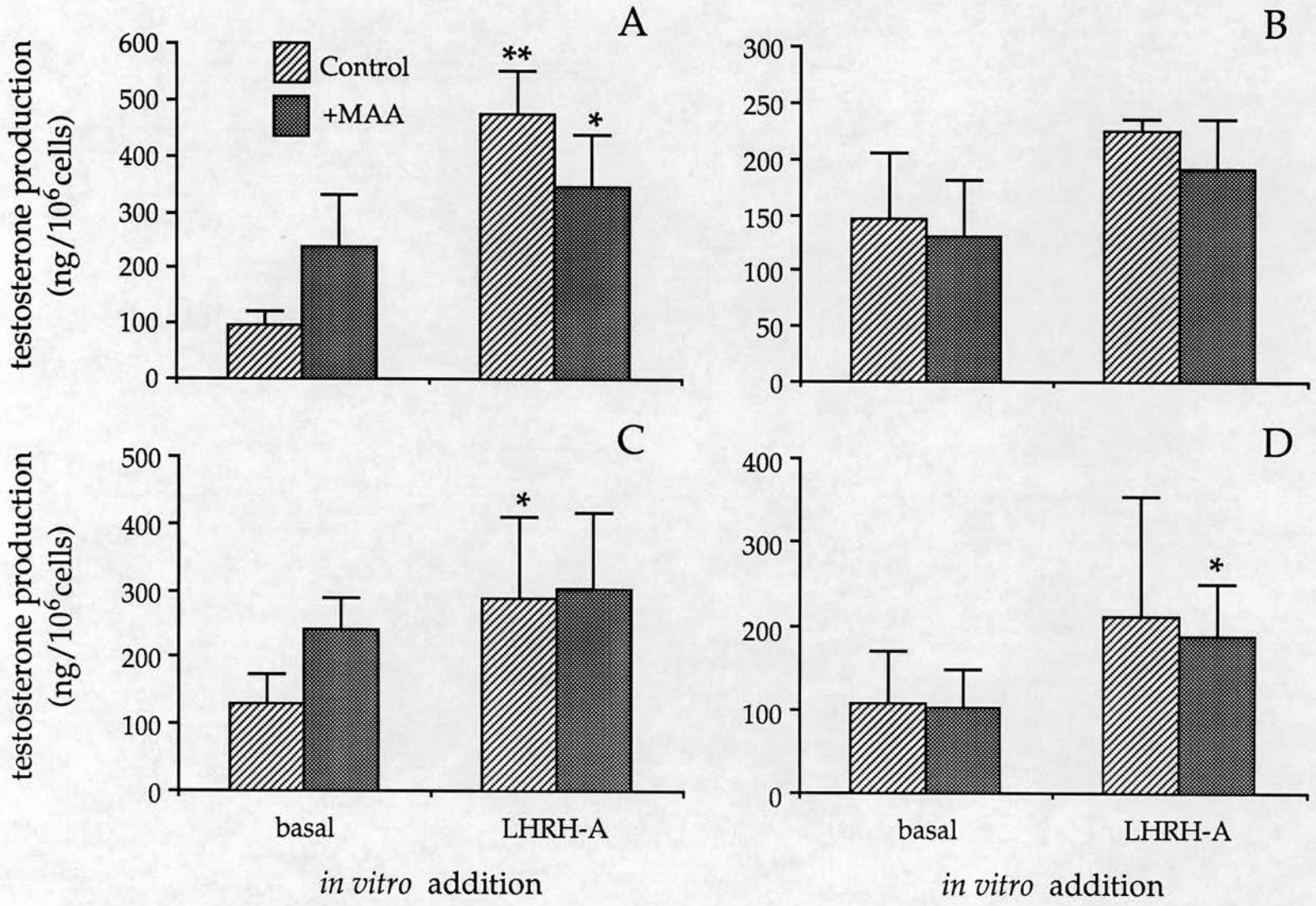

Control
+ MAA
testosterone production
(ng/10^6cells)
1500
1250
1000
750
500
250
0
0
10
20
30
40
50
days after MAA treatment

Figure 7.3. The effects of LHRH-A on basal testosterone secretion by rat Leydig cells isolated from control and MAA-treated rats at successive intervals after treatment.

The graph shows basal testosterone secretion in response to LHRH-A in control (o) and treated (•) rats. **A.** 3 days post-MAA administration. **B.** 7 days post-MAA administration. **C.** 14 days post-MAA administration. **D.** 21 days post-MAA administration. Each point is the mean±S.D of 3 separate experiments. $*p < 0.05$, $**p < 0.01$, effect of LHRH-A significantly different from the appropriated control.


A
B
C
D
Control
+MAA
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
600
500
400
300
200
100
0
300
250
200
150
100
50
0
500
400
300
200
100
0
400
300
200
100
0
**
*
*
*
basal
LHRH-A
basal
LHRH-A
in vitro addition
in vitro addition

7.4. Discussion

The experiments described in this chapter were designed to use *in vivo* depletion of specific germ cell types to examine *in vitro* the potential cascade of cell-cell interactions whereby germ cells acting through Sertoli cells might exert an influence on Leydig cell function. Also, LHRH agonists are widely considered to have a potential paracrine role in the rat, and possibly the human, testis (see chapter 6). Thus the effect of *in vivo* germ cell depletion on the *in vitro* response of Leydig cells to this potential paracrine modulator was investigated.

In the rat there is growing evidence to support the existence of functional interactions between the various cell types and compartments of the testis (for reviews see Sharpe, 1986, Tahka, 1989, Sharpe, 1990, Skinner, 1991). For instance it has been shown that addition of enriched preparations of particular germ cell types (PS or RS) to immature Sertoli cell cultures modulates the secretion of Sertoli cell products such as oestradiol (Jegou *et al.*, 1988), transferrin (Le Magueresse *et al.*, 1988) and ABP (Galdieri *et al.*, 1984). It is also clear that Sertoli cell function changes according to the stage of the spermatogenic cycle (Parvinen, 1992) and this is considered to be a consequence of the changing germ cell complement at each stage (Sharpe, 1986). In support of this hypothesis it has been shown that there is a rapid decline in the secretion of a number of Sertoli cell products following partial or major depletion of particular germ cells from the seminiferous epithelium. Vihko *et al.* (1984) demonstrated that selective depletion of preleptotene spermatocytes by X-irradiation of the testes abolished the peak secretion of plasminogen activator which occurs at stages VII-VIII in the normal testis. This abolition was not seen when other classes of germ cell were depleted. Similarly, Allenby *et al.* (1991) showed that the rate of secretion of immunoactive inhibin by Sertoli cells was modulated by the depletion of ES (and possibly PS), while the depletion of other germ cell types had no effect on inhibin secretion. Recently it has been demonstrated that co-culture of Sertoli cells with either PS or RS increases the expression of the preproenkephalin gene by Sertoli cells (Fujisawa *et al.*, 1992). Altogether results such as these imply that there is active communication between germ cells and Sertoli cells.

Available evidence also indicates the existence of Sertoli cell-Leydig cell interactions. Factors which probably derive from Sertoli cells have been shown to alter Leydig cell testosterone secretion *in vitro*. Rat Sertoli cells are thought to produce 'testicular LHRH' (Sharpe *et al.*, 1981) and Leydig cells are the only testicular cells known to have LHRH receptors (Bourne *et al.*, 1980). Also, addition of Sertoli cell conditioned medium to Leydig cell cultures enhances testosterone production (Janecki *et al.*, 1985, Verhoeven & Cailleau, 1985, Papadopoulos *et al.*, 1987b, Grootenhuis *et al.*, 1990). In the rat, Sertoli cell secreted proteins have also been shown to stimulate Leydig cell replicative DNA synthesis *in vitro* (Ojeifo *et al.*, 1990).

Recently Onoda *et al.* (1991) demonstrated that the influence of germ cells on other testicular cell types extended beyond the seminiferous epithelium into the interstitium to affect Leydig cell function. Cultured Sertoli cells were shown to secrete a factor(s) which stimulated Leydig cell steroidogenesis and PS were shown to inhibit the secretion of this factor(s) from Sertoli cells. While this is the first direct evidence of an interaction between germ cells, Sertoli cells and Leydig cells, indirect evidence of such a relationship has been accumulating for over a decade.

In 1978 Aoki & Fawcett showed how testicular implants of cyproterone acetate caused disruption of the seminiferous epithelium in tubules within a radius of a few mm of the implant. All other tubules were unaffected and Leydig cell hyperplasia occurred only adjacent to the damaged tubules. These local effects on Leydig cells were independent of changes in the level of serum gonadotrophins (changes which would have affected *all* Leydig cells) and it was proposed that damage to the seminiferous epithelium had interfered with the production of a factor(s) with a regulatory action on Leydig cells. Similarly, induction of unilateral testicular damage by cryptorchidism caused changes in Leydig cell and Sertoli cell function only in the testis in which spermatogenesis had been disrupted, again negating the influence of serum gonadotrophins and raising the possibility that seminiferous tubules can influence Leydig cell function (Risbridger *et al.*, 1981). This idea is supported by the work of Bergh (1983, 1985), who demonstrated that Leydig cells adjacent to stage VII-VIII tubules were significantly larger than those proximal to other stages.

Using heat exposure of the testis to induce disruption of spermatogenesis, Jegou *et al.* (1984) found that changes in Leydig cell function were separated in time from the actual thermal stress but occurred in association with the depletion of a specific germ cell type (elongated spermatids). The implication was that the effects observed were a consequence of changes in Sertoli cell function, secondary to disruption of germ cell function. Papadopoulos *et al.* (1987) found that addition of either Sertoli cell conditioned medium or seminiferous tubule conditioned medium to cultured Leydig cells stimulated testosterone production. However, if the seminiferous tubule conditioned medium was collected from γ-irradiated rats, the enhancement of Leydig cell steroid output was even greater. Thus a germ cell-Sertoli cell interaction modulates the action of Sertoli cell factors on Leydig cells.

To date, in order to study paracrine interactions in the testis it has been necessary to work with isolated cells or co-cultures. This is not the most satisfactory approach now that it appears that paracrine interactions co-ordinate the functions of all testicular cell types. An alternative approach in the study of this cascade of cell-cell interactions is to use MAA to remove specific germ cell populations *in vivo* (Bartlett *et al.*, 1988), leaving the majority of cells, and their interactions, intact. Thus it has been possible to expand on the work of Onoda *et al.* (1991), and use an *ex vivo* model to demonstrate germ cell-Sertoli cell-Leydig cell interactions.

It was found that the *in vivo* absence of either a majority of PS (d3 post-MAA) or RS (d14 post-MAA) caused an increase in the basal level of testosterone production by isolated Leydig cells in comparison with the respective controls. As only basal testosterone levels were affected by the administration of MAA it was possible that raised LH levels *in vivo* had resulted in the increased testosterone production seen *in vitro*. However, it has been demonstrated previously that serum LH levels are not significantly affected by MAA-treatment except at days 21 and 28 post-administration, when an increase is seen. At 21 days after MAA-treatment, the *in vivo* absence of ES had no effect on *in vitro* testosterone production. Serum FSH levels are affected by MAA administration, but the pattern of increases is not consistent with the changes in Leydig cell function described (Allenby *et al.*, 1991).

At d7 post-MAA treatment when an equal number of tubules were missing PS as were missing RS, there was no difference in testosterone production in Leydig cells isolated from control or MAA-treated animals. This was a surprising finding considering the results obtained at d3 and d14 post-MAA. In a previous study, Bartlett *et al.* (1988) found an increase in serum FSH 3 days after MAA treatment, FSH levels then returned to control values before reaching a second peak at 21 days post-MAA treatment. Levels of ABP in interstitial fluid followed an identical pattern. The authors suggested that this biphasic response to MAA treatment was related to the germ cell type missing from the epithelium of the majority of tubules after treatment, and/or, to the stage of spermatogenesis of affected tubules. This would be consistent with the known variations in Sertoli cell function with the stage of the cycle of the seminiferous epithelium (Parvinen, 1992). In the experiments presented here, the first explanation does not seem likely considering the similarity in germ cell types missing between tubules from days 3 and 7 (PS) and from days 7 and 14 (RS) post-MAA treatment. The types of germ cells missing from each stage of the spermatogenic cycle at 3, 7 and 14 days after MAA administration are shown in Table 7.1. If the effects described in Figure 7.1. are related to alterations in germ cell complement at specific stages, then the complement of any stage at d3 and d14 post-MAA, when there is an *in vitro* effect, must be different from that at d7 post-MAA, when there is no *in vitro* effect. In such a stage the germ cell complements at d3 and d14 post-MAA do not necessarily have to be the same. Only two stages (I and VII, highlighted in Table 7.1.) meet this criteria. At stage VII the differences between days 3, 7 and 14 post-MAA are in absolute numbers, rather than the type of germ cell missing, which is a very specific difference. If the hypothesis that the stage of spermatogenesis in affected tubules is important in germ cell-Sertoli cell-Leydig cell interactions is correct, it would allow the further hypothesis that seminiferous tubules at stage I act through Sertoli cells (presumably) to suppress Leydig cell steroidogenesis, and when stage I tubules are damaged in any way, the production of this inhibitory factor is suppressed or prevented and the basal level of testosterone production increases.

The disruption of the seminiferous epithelium described in this chapter, causes, at particular time points, an increase in testosterone

production by isolated Leydig cells. Aoki & Fawcett (1978) also found that localised seminiferous tubule damage caused an increase in Leydig cell activity, specifically, hyperplasia. Jegou *et al.* (1984) demonstrated that at particular time points after the testis had been subjected to short-term heating, there was an increase in testosterone production *in vitro* by decapsulated testes. Sharpe *et al.* (1986), showed that seminiferous tubule dysfunction consequent to EDS administration, caused an increase in the bioactivity of interstitial fluid which in turn would cause an increase in Leydig cell steroid production. Thus it appears that damage to the seminiferous epithelium, in particular damage to the germ cell population, results in an increase in the activity of Leydig cells, suggesting that germ cells normally secretes a factor(s) which negatively regulates Sertoli cell modulation of Leydig cell function.

The *in vitro* work of Onoda *et al.* (1991), supports this theory. The authors showed that cultured Sertoli cells secrete a protein(s) that stimulates Leydig cell steroidogenesis. These results suggest that Sertoli cells normally secrete a stimulatory factor. Addition of PS proteins suppressed the secretion of this stimulatory factor. The suppressive effect on Leydig cell steroidogenesis of PS proteins would be in accordance with the germ cell effects described above.

In the presence of a maximally stimulating dose of hCG, such marked between-experiment variation occurred that it was impossible to dissect out any effect of MAA administration (ANOVA; $p = 0.688$, Figure 7.2). When the effects of potential paracrine modulators of Leydig cell function are studied *in vitro*, it is often the case that when a compound has an effect on basal testosterone production, it has no effect on hCG-stimulated testosterone production. This is the case with both AVP and LHRH-A (see Chapter 6 for references). It is possible that paracrine effects are swamped by the addition of such supraphysiological concentrations of hCG.

LHRH-A has a stimulatory effect on testosterone production by cultured Leydig cells (see Chapter 6). At those time points where *in vivo* administration of MAA had not affected Leydig cell testosterone production *in vitro*, namely days 7 and 21 post-MAA, there was no difference in the response to LHRH-A of Leydig cells isolated from control and treated animals (Figure 7.3 B and D). However, on days 3 and 14 after

MAA treatment, there was a decline in the fold responsiveness to LHRH-A between Leydig cells isolated from controls (p <0.01 on d3, p <0.05 on d14) and Leydig cells isolated from MAA treated animals (p <0,05 on d3, not significant at d14). The decrease in fold responsiveness was a consequence of the increase in basal testosterone output which was not matched by a similar increase in stimulation when LHRH-A was added to the culture system. There are several possible explanations of this observation. It is possible that the germ cell effects seen at d3 and d14 post-MAA are either mediated through LHRH receptors or use a common second messenger system. Alternatively, the effects mediated by germ cells could result in the down-regulation of LHRH receptors. This explanation would mean that the stimulatory effect of LHRH (seen *in vitro*) would be weakened, thereby eliminating a signal that would counteract the inhibitory effect of the germ cell modulated signal.

In summary, the experiments presented in this chapter have shown how germ cells, presumably acting via Sertoli cells, are able to influence the production of testosterone by Leydig cells. Moreover, this regulatory effect was shown to be mediated by specific germ cell types, that is, by PS and RS, and there was a further suggestion that the modulation was also stage-dependent, possibly being orchestrated by stage I tubules.

Table 7.1. Representation of the germ cells depleted from each stage of the spermatogenic cycle at 3, 7 and 14 days after treatment with MAA.

PS = pachytene spermatocytes, RS = round spermatids, ES = elongate spermatids, N = normal germ cell complement.

stage of spermatogenesis	days post-MAA administration		
	3	7	14
I	PS/RS	N	RS
II	PS	RS	RS
III	PS	RS	RS
IV	PS	RS	RS
V	PS	RS	RS
VI	PS	RS	RS
VII	PS	PS/RS	RS
VIII	N	PS	N
IX	N	PS	ES
X	N	PS	ES
XI	N	PS	ES
XII	PS	PS	ES
XIII	PS	PS	ES
XIV	PS	PS	ES

8. Leydig cell proteins

The previous chapter demonstrated how cell-cell interactions can occur in the testis such that changes in the germ cell population of the seminiferous epithelium can indirectly affect the function of Leydig cells in the interstitium. It was shown that when PS or RS were the major germ cell types missing, Leydig cell testosterone production was increased in comparison to controls. However when approximately equal numbers of PS and RS were depleted, no change was seen in Leydig cell testosterone production. These apparently discordant results may possibly be related to germ cell damage at particular stages of the spermatogenic cycle. The experiments described in this chapter concern Leydig cell protein production and how it may be influenced by the above alterations in germ cell population, with the aim of identifying proteins with potential as paracrine regulators, involved in the interactions between the seminiferous epithelium and the interstitium.

8.1. Introduction

Cell-cell interactions appear to form an integral part of testicular physiology (Skinner, 1991), with interactions occurring both within and between testicular compartments. The influence of the seminiferous epithelium on Leydig cell function is well documented (Sharpe, 1990, 1992). Conversely, the influence that Leydig cells have on the seminiferous epithelium is absolute as it is clear that the production of testosterone is essential for normal spermatogenesis (Sharpe, 1987). However, it is not clear what contribution non-steroidal Leydig cell products make towards the normal functioning of the testis. Sharpe *et al.* (1988) demonstrated that when rat testes were depleted of Leydig cells by administration of EDS, but supplemented with testosterone esters, quantitatively normal spermatogenesis could be maintained for a period of at least 10 weeks. This suggested that Leydig cell products other than testosterone were not essential for the maintenance of spermatogenesis

and fertility in the rat. However, in testes depleted of Leydig cells but exposed to exogenous testosterone, there was a 3-4 fold increase in the incidence of germ cell degeneration occurring in stages XIV-I. These changes were not seen in control rats supplemented with testosterone esters and the change was therefore assumed to be dependent on he absence of Leydig cells.

In the absence of Leydig cells no dialogue can occur between the epithelium and the interstitium, so while the presence of testosterone ensures the continuation of spermatogenesis, the interactions which might create the optimal conditions for spermatogenesis do not occur, possibly resulting in the noted increase in germ cell degeneration. The results presented in chapter 7 demonstrated how the absence of PS and RS *in vivo*, can increase testosterone production *in vitro*, an effect which appeared to be limited to germ cell depletion at stage I of the spermatogenic cycle. Thus the study by Sharpe *et al.* (1988) associated pharmacological levels of intratesticular testosterone with increased germ cell degeneration at stages XIV-I, while the results presented in chapter 7 associated germ cell damage at stage I with increased testosterone production. Together these studies are strongly indicative of a bidirectional interaction between Leydig cells and the seminiferous epithelium, which may act to regulate the amount of testosterone produced by Leydig cells.

Few non-steroidal Leydig cell secretory products have been identified, but those that have been characterised (POM-C peptides, IGF-1, inhibin, Skinner, 1991) do appear to be involved in cell-cell communication, and are therefore potential mediators of the interaction described above. To test the hypothesis that a Leydig cell protein might mediate an important cell-cell interaction, the pattern of Leydig cell proteins secreted under different culture conditions was investigated. Protein secretion was assessed by measuring ^{35}S-methionine incorporation into newly produced secreted proteins. The M_r and pI of the proteins secreted by Leydig cells were found by analysis of autoradiographs of 2-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein production under basal and hCG-stimulated culture conditions was compared. In addition, proteins secreted by Leydig cells isolated from rats with PS and

RS absent from stage I were also examined, to assess the influence of the epithelium on non-steroidal Leydig cell products.

In the study by Sharpe *et al.* (1988), the authors state, that although in the rat the absence of Leydig cell products other than testosterone had a negligible effect on sperm output and fertility, they might have more relevance in less fertile species, such as man, in which degeneration of spermatocytes in the final stages of meiosis (i.e. stages XIV-I) is estimated to cause a 40% reduction in the potential yield of spermatids (Johnson *et al.*, 1984). With this in mind a comparison was made between the types of proteins secreted by rat and human Leydig cells *in vitro*.

8.2. Experimental Procedures

8.2.1. Leydig cell culture conditions

Leydig cells were isolated according to the methods described in chapter 3. Cells were plated out at 100,000 cells per well in 300µl culture medium. After a 2h preincubation period, the medium was removed and replaced with Eagles minimal essential medium without methionine (I.C.N. Flow). This was supplemented with 4 nmol/L of L-glutamine, 100U penicillin/ml, 100µg/ml streptomycin, 25mmol/L Hepes (Gibco), and 0.1% polyvinyl alcohol (Sigma). Prior to incubation 60µCi ^{35}S-methionine (Amersham International, Amersham, England) was added to give a final concentration of 0.75µM methionine/well. Cells were incubated in the presence or absence of 30U/ml hCG for 20h, then the medium was aspirated and stored at -20°C until required.

8.2.2. Measurement of newly synthesized proteins by trichloroacetic acid (TCA) precipitation.

10µl of sample was incubated on ice for 15-30 mins in the presence of 10µl 0.1M PBS + 1%BSA, and 1ml 10% TCA + 10mM methionine. The solution was then centrifuged for 5 mins at 13000rpm to pellet the precipitate. The supernatant was discarded and the pellet washed in 200µl 0.1M potassium hydroxide. A second precipitation step was performed by the addition of 1ml 10% TCA + 10mM methionine for a further 15-30

mins on ice. The solution was centrifuged at 13000rpm for 5 mins and washed in 0.4M potassium hydroxide. The ^{35}S-methionine content of the precipitate was counted in an NE 1600 (New England Nuclear, USA) gamma counter.

8.2.3. Isoelectric focusing (first dimension separation).

The gel mix was prepared by degassing a solution consisting of 5.5g urea (Sigma), 1.33ml 30% acrylamide (Bio-Rad, Hemel-Hempstead, England), 2.15ml deionised distilled ('clean') water, 2ml 10% nonidet P-40 (BDH), and 0.3ml pH 5-7 biolyte ampholines (Bio-Rad), 0.15ml pH 7-9 ampholines and 0.15ml pH 3-10 ampholines. Polymerisation was initiated by the addition of 10µl 10% ammonium persulphate (Sigma) and 5µl TEMED (Sigma). The tube gels were cast to a height of 12cm in 160mm x 2.5mm internal diameter glass tubes (Bio-Rad), and overlaid with 1cm of clean water and left for approximately 2h to polymerise. The water was then replaced with 20mM NaOH and left for a further hour. Before mounting the gels on the electrophoresis unit (Protean II, Bio-Rad), the NaOH at the top of the gels was replaced with degassed NaOH. The bottom of the electrophoresis tank was filled with 4l 6mM phosphoric acid (anolyte), and the core assembly containing the tube gels was lowered into this solution. Care was taken to remove air bubbles from the ends of the tube gels. Water cooling pipes were attached and the top tank filled with degassed 20mM NaOH (catholyte). The gels were prefocused at 200V for 15 mins (3000xi power supply; Bio-Rad), then at 300V for 30 mins and 400V for 30 mins.

Samples were prepared for loading by the addition of an equal volume of sample buffer (9.5M urea, 2% nonidet P-40, 2% ampholines and 1% dithiothreitol) and enough urea to readjust the final concentration to 9.5M (28mg urea : 50µl sample). Each such sample mixture was incubated at room temperature for 15 mins, centrifuged at 13000 rpm for 3 minutes and the supernatant loaded onto the tube gels. Each gel was loaded with equal cpm (250-350,000) radiolabelled protein. In order to measure the pH gradient created by the ampholines, blank gels were loaded with 60µl of sample buffer alone. Gels were focused at 400V for 14h, followed by 2h at 800V.

Gels were extruded from the glass tubes using water pressure, then incubated in equilibration buffer [0.5M Tris-HCl, pH 6.8, 10% sodium dodecyl sulphate (SDS; Sigma), 0.05% bromophenol blue (Bio-Rad)] for 3 mins at room temperature. The gels were frozen rapidly in dry ice/ethanol, and stored at -40°C. The blank gels were cut into 24x5mm segments each of which was placed in 1.4ml clean water. The pH of the water was measured 4 and 6h later.

8.2.4. Protein separation on the basis of molecular weight (second dimension separation)

Slab gels were prepared by degassing a solution consisting of 40mls 1.5M Tris-HCl, pH 8.8, 58.65ml 30% acrylamide and 60.15ml clean water. This amount is sufficient for 4 gels. The glass plates used to make the gels were cleaned with pyroneg, washed thoroughly in clean water and polished with ethanol. Polymerisation of the gel mix was initiated by the addition of 1.2ml 10% ammonium persulphate and 36µl TEMED. Once poured the gels were overlaid with water-saturated secondary butanol (Aldrich), and left for a maximum of 1.5h to polymerise. The butanol was then removed and the top of the gel washed thoroughly with clean water. If the gels were for immediate use they were overlaid with separating gel buffer (1.5M Tris-HCl diluted 1:4) for a further 2-3h. If the gels were for use on the following day, then once the separating gel buffer was in place the top of the gel cast was sealed and the unit stored at 4°C overnight. The next step was to remove the buffer and wash the gel thoroughly with clean water. 1% agarose (Sigma) was used to make wells for the molecular weight markers. Frozen tube gels were thawed at room temperature then positioned carefully on the top of the slab gel. Care was taken to ensure tight contact between the tube gel and the slab gel; all air bubbles were carefully removed. Molecular weight markers (Bio-Rad) were loaded. The running buffer used consisted of 0.3% Tris base (Sigma), 1.44% glycine (Sigma) and 0.1% SDS in clean water. The gels were run at either 38mA/gel (constant current) for 2.5h, or overnight at 25mA/gel for 10 mins, then 5mA/gel overnight and 40mA/gel for 15 mins.

8.2.5. Silver staining

Gels were fixed in 40% methanol, 10% acetic acid for a minimum of 30 mins followed by 10% ethanol, 5% acetic acid, also for 30 mins. The gels were then stained using the Bio-Rad silver staining kit; gels were washed in oxidising solution for 5 minutes and then washed in clean water for 2 periods of 30 mins. They were then washed in silver staining solution for 20 mins followed by a short rinse in clean water. Developer was added until a smokey precipitate appeared, the solution was then removed and replaced with fresh developer for a further 5 mins. The developing solution was replaced again, and the fresh developer was left on the gels until they appeared to be optimally stained. The developing reaction was stopped by the addition of 5% acetic acid, and the gels were either stored in clean water or dried down immediately. Prior to drying gels, were washed for 20 mins in Amplify (Amersham), they were then dried for 2h at 62°C on a model 543 drier (Bio-Rad).

8.2.6. Developing Autoradiographs

Dried gels were exposed to X-ray film (Kodak X-OMAT ARS) at -80°C for 21-28 days. Autorads were allowed to warm to room temperature before being developed. To do this the film was placed in developer (Kodak) for 4 mins, washed thoroughly with tap water and then fixed (Kodak fixative), washed again and allowed to dry.

8.3. Results

8.3.1. Secretion of newly synthesized proteins.

There was no significant difference in the incorporation of ^{35}S-methionine into newly secreted Leydig cell proteins cultured in the presence or absence of hCG (Figure 8.1). Leydig cells isolated from rats treated 3, 7 or 14 days earlier with MAA also showed no difference in the level of ^{35}S-methionine incorporation into newly secreted proteins (Figure 8.2).

8.3.2. Patterns of protein secretion.

Analysis by 2-D SDS-PAGE of newly synthesized proteins secreted by rat Leydig cells in the presence and absence of hCG is shown in Figure 8.3. It can be seen that Leydig cells *in vitro* secrete over 70 proteins. There are no obviously different proteins secreted by Leydig cells cultured in the presence or absence of hCG. The proteins or protein groups labelled on the gels have similar M_rs to proteins which are known Leydig cell products. The pregnancy specific β_1-glycoproteins (PSG) are a heterogeneous family of proteins which have been localized to Leydig cells in the rat testis (Ogilvie *et al.*, 1990, Richardson *et al.*, 1991). PSGs have M_rs in the 90kDa range, and protein group 1 has M_rs in this range. Leydig cells also secrete the calcium binding protein named, secreted protein, acidic and rich in cysteine (SPARC; Vernon & Sage, 1989). The M_r of SPARC is 43kDa, coincident with that of protein 2. The 3 proteins comprising group 3 have similar M_rs (30kDa) to the 3 forms of platelet derived growth factor (PDGF) which are found in rat Leydig cells (Gnessi *et al.*,1992).

Leydig cells isolated on different days (3, 7, 14) following administration of MAA, and cultured under basal conditions did not show any major differences in the proteins they secreted (Figure 8.4.). There was one basic protein, with an apparent M_r of 35kDa that was secreted by Leydig cells isolated on days 3 and 14 after MAA treatment that did not appear to be secreted by Leydig cells isolated at 7 days after MAA treatment (protein 4). This protein was not secreted by Leydig cells isolated from control animals and cultured under basal conditions (Figure 8.4.), but Leydig cells isolated from control animals and cultured in the presence of hCG appeared to secrete small amounts of this protein (Figure 8.4.).

There was a remarkable similarity in the proteins secreted by rat and human Leydig cells *in vitro* (in the absence of hCG), at least as judged by M_r and pI. On this basis, nine potentially homologous proteins or protein groups have been identified (Figure 8.5.). Of these proteins 5 and 9 appeared in equal concentrations in rat and man. Proteins 1 and 4 appeared to be more abundant in the rat, however the majority of these

proteins were produced to a greater extent by human Leydig cells compared to the rat.

8.4. Discussion

The experiments described in this chapter examined Leydig cell protein production, and how this is influenced by the seminiferous epithelium. The preceding chapter demonstrated that altering the normal germ cell complement *in vivo* affected Leydig cell testosterone production *in vitro*. Surprisingly, the relationship between germ cells and Leydig cells seemed to be confined to germ cells at a specific stage of the spermatogenic cycle. In this chapter the influence of germ cells on Leydig cell protein secretion was investigated. Also, a comparison was made of the proteins secreted by rat and human Leydig cells *in vitro*.

Analysis of proteins secreted by Leydig cells *in vitro* has shown that neither the incorporation of ^{35}S-methionine into newly secreted proteins, nor the actual proteins produced, were affected greatly by *in vitro* exposure to hCG or by the *in vivo* depletion of specific types of germ cell prior to Leydig cell isolation. The *in vivo* absence of either PS or RS at days 3 and 14 after treatment with MAA, while not affecting the overall level of protein secretion by isolated Leydig cells, resulted in the secretion of a 35kDa basic protein which was not secreted by Leydig cells isolated from either control animals, or animals 7 days after MAA administration. This protein also appeared to be secreted in small quantities by Leydig cells isolated from control animals but cultured in the presence of hCG.

In general, non-steroidal Leydig cell products have not been thoroughly investigated and to date most of the work on Leydig cell protein production relates to intracellular proteins. These proteins are not secreted into the interstitium but have potential roles as intracellular modulators of steroidogenesis. Themmen *et al.* (1985, 1986) described how LH and PMA stimulated the phosphorylation of 17 and 33 kDa proteins, which are believed to be part of the mechanism by which LH mediates its effects. Leydig cells have also been found to contain a 28kDa protein the production of which is induced by oestrogen. This protein is also speculated to have a role in intracellular regulation of steroidogenesis

Figure 8.1. The effect of hCG on the incorporation of ^{35}S-methionine into newly synthesized proteins secreted *in vitro* by rat Leydig cells.

Each column is the mean±S.D. of 9 separate experiments.

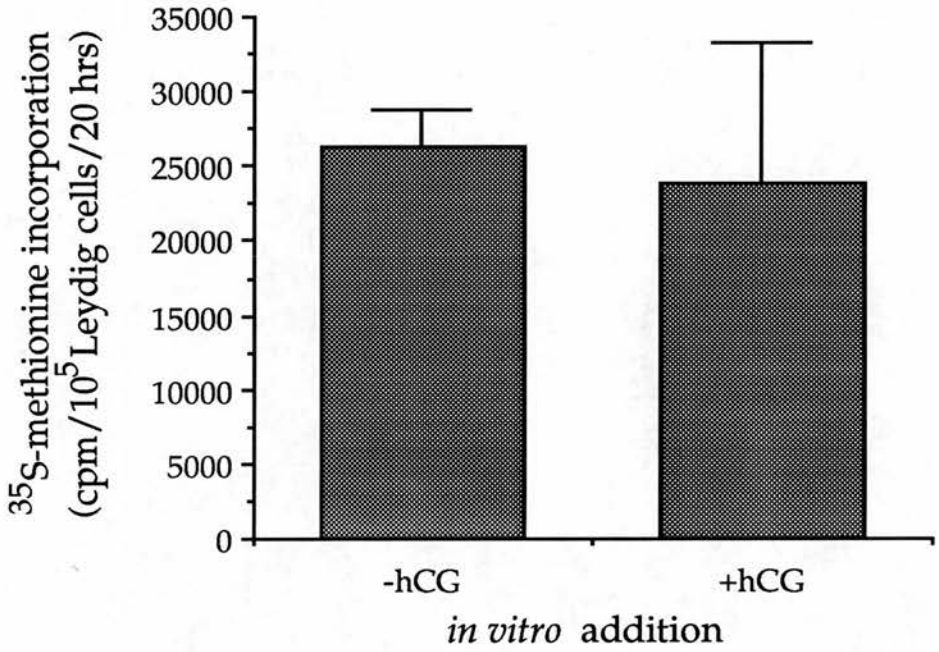

35000
30000
25000
20000
15000
10000
5000
0
^{35}S-methionine incorporation
(cpm/10^5 Leydig cells/20 hrs)
-hCG
+hCG
in vitro addition

Figure 8.2. The effects of administration of MAA on the incorporation of ^{35}S-methionine into newly synthesized proteins secreted *in vitro* by rat Leydig cells at successive intervals after treatment.

Each column is the mean±S.D. of either 3 (days 3 and 7) or 2 (day 14) separate experiments .

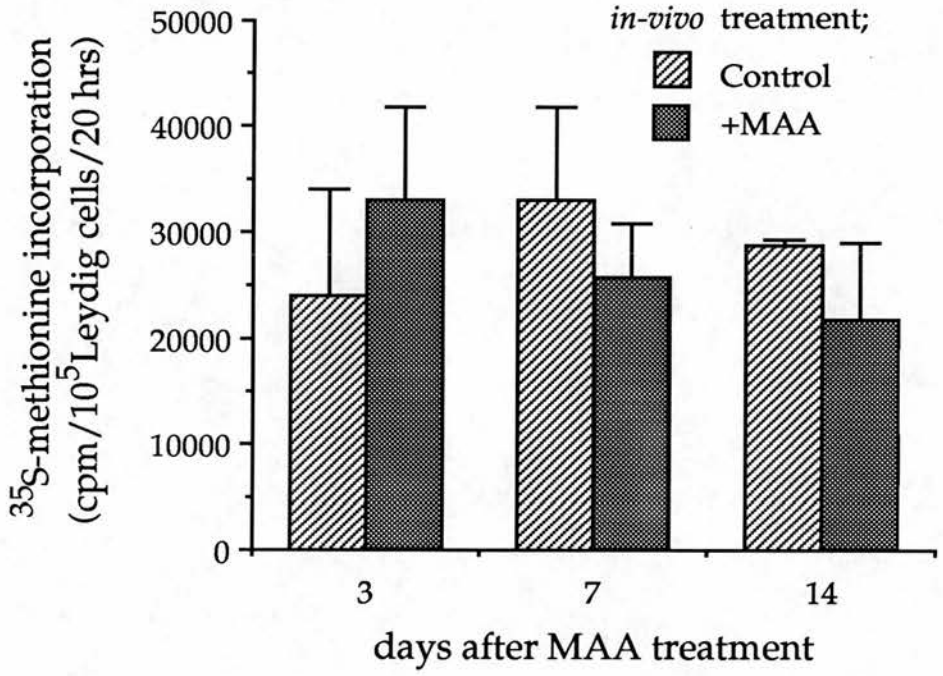

50000
40000
30000
20000
10000
0
^{35}S-methionine incorporation
(cpm/10^5Leydig cells/20 hrs)
in-vivo treatment;
Control
+MAA
3
7
14
days after MAA treatment

Figure 8.3. Comparison of the pattern of proteins secreted by isolated rat Leydig cells in the absence (top) or presence (bottom) of hCG during a 20h incubation.

Comparable fluorograms were obtained on 5 (minus hCG) and 3 (plus hCG) separate occasions. Proteins/protein groups which have comparable M_rs to known Leydig cell products have been circled and numbered. Protein group 1 has a comparable M_r to PSG, protein 2 has a comparable M_r to SPARC and protein group 3 has a comparable M_r to PDGF. Protein 4 is secreted only in the presence of hCG.

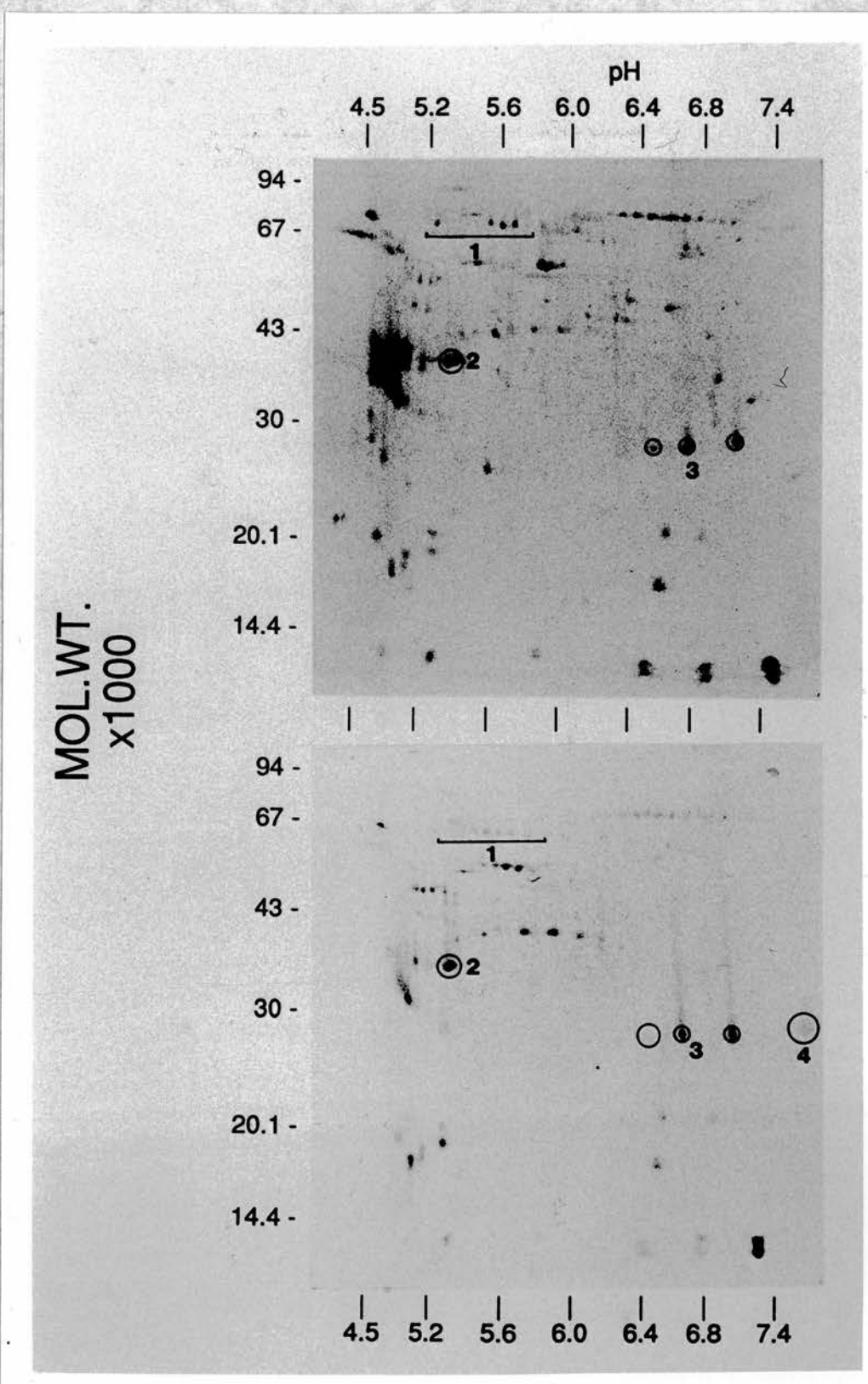

pH
4.5
5.2
5.6
6.0
6.4
6.8
7.4
94 -
67 -
43 -
30 -
20.1 -
14.4 -
1
2
3
4
MOL.WT.
x1000

Figure 8.4. Comparison of the pattern of proteins secreted *in vitro* by Leydig cells isolated from the testes of adult rats at various times after administration of MAA.

A. control (no MAA administered). **B.** 3 days after administration of MAA. **C.** 7 days after administration of MAA. **D.** 14 days after administration of MAA. Comparable fluorograms were obtained on 2 separate occasions. Proteins/protein groups which have comparable M_rs to known Leydig cell products are circled and numbered. Protein group 1 has a comparable M_r to PSG, protein 2 has a comparable M_r to SPARC and protein group 3 has a comparable M_r to PDGF. Protein 4 was secreted 3 and 14 days after administration of MAA, but was not secreted 7 days after administration of MAA.

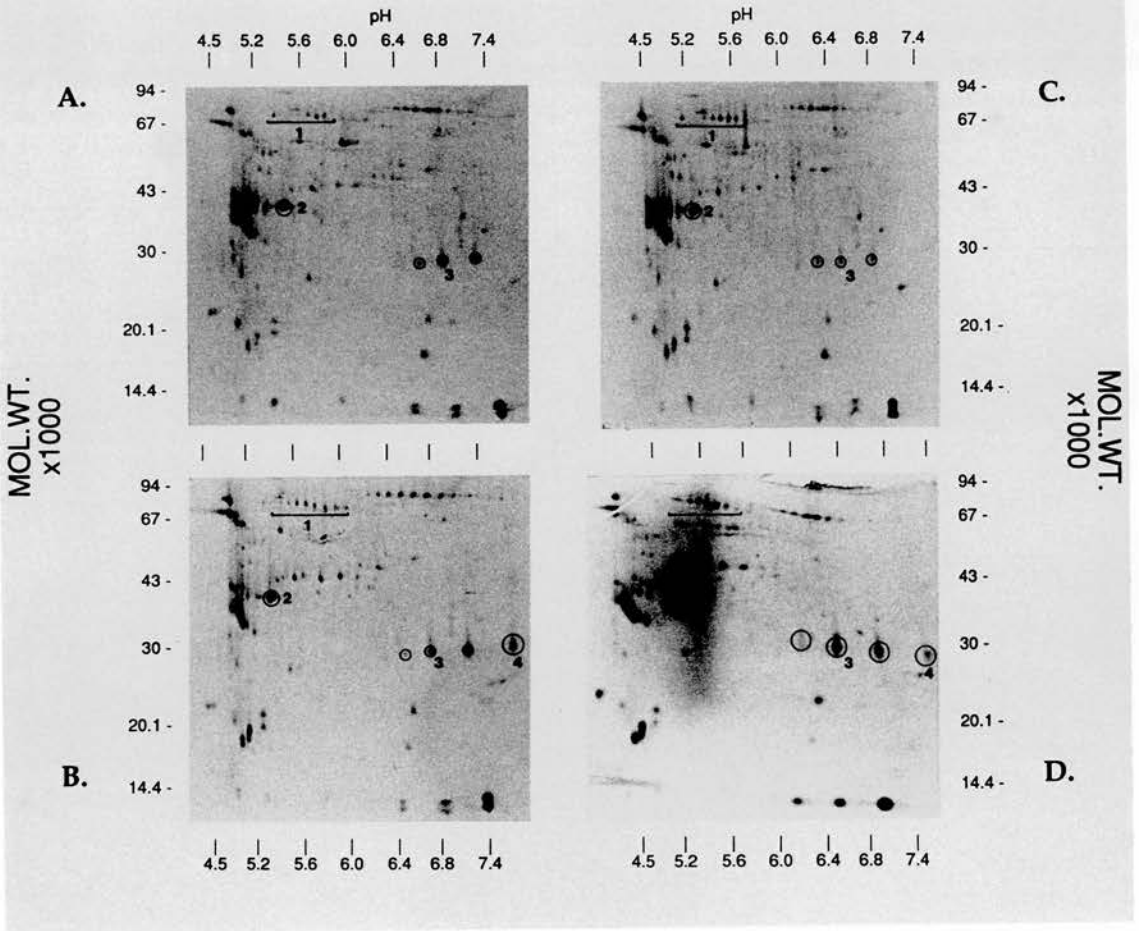
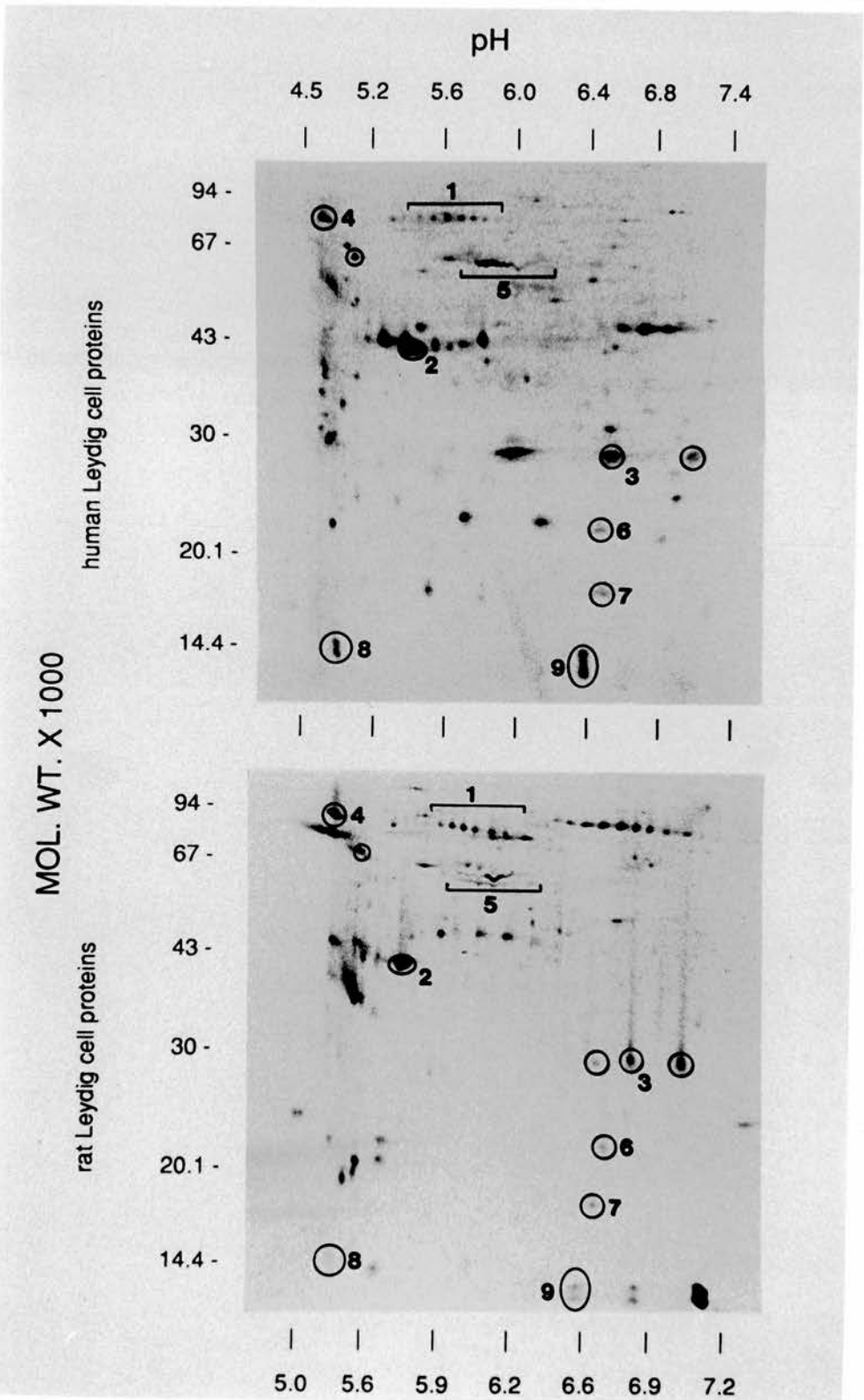

A.
B.
C.
D.
pH
4.5 5.2 5.6 6.0 6.4 6.8 7.4
MOL.WT.
x1000
94 -
67 -
43 -
30 -
20.1 -
14.4 -
1
2
3
4

Figure 8.5. Comparison of the pattern of proteins secreted by isolated human (top) and (rat) Leydig cells during a 20h incubation.

Proteins/protein groups with comparable M_rs and pIs have been circled and numbered. They include PSG (1), SPARC (2) and PDGF (3). Comparable fluorograms were obtained on 5 (rat) and 2 (human) separate occasions.


pH
4.5
5.2
5.6
6.0
6.4
6.8
7.4
94 -
67 -
43 -
30 -
20.1 -
14.4 -
1
2
3
4
5
6
7
8
9
human Leydig cell proteins
rat Leydig cell proteins
MOL. WT. X 1000
5.0
5.6
5.9
6.2
6.6
6.9
7.2

(Ciocca *et al.*, 1986). One of the best characterised intracellular Leydig cell proteins is sterol carrier protein$_2$ (SCP_2). The effect of SCP_2 *in vitro* is to accelerate the transfer of phospholipid and cholesterol between membranes (van Noort *et al.*, 1988), thus facilitating steroidogenesis.

Little is known about the proteins which are secreted by Leydig cells, although it is possible that such proteins may influence Sertoli cell function. The importance of paracrine interactions in the testis has been discussed in previous chapters and has been extensively reviewed (for example, Skinner, 1991, Sharpe, 1992), although Ratnasooriya & Sharpe (1989) questioned the importance of Leydig cell products other than testosterone when they described how spermatogenesis could be maintained for a 10 week period in testes devoid of Leydig cells but supplemented with testosterone. While few Leydig cell secretory products have been identified or characterised, those that have, such as the POM-C peptides, inhibin and IGF-1, are thought to be involved in cell-cell interactions (Skinner, 1991). Another protein secreted by Leydig cells is testicular immunosuppressive protein, thought to be involved in conferring immune privilege on the testicular interstitium (Pollanen *et al.*, 1988). However, with an M_r of 130kDa, testicular immunosuppressive protein is too large to be resolved on the gels used in these experiments. Proteins which are known to be secreted by Leydig cells *in vitro* and which do have an M_r within the limits defined by the gels include interleukin-1 (IL-1; 17kDa, pI 5.5, Bellve & Zheng, 1989), nerve growth factor (NGF; 13kDa, pI 9.3, Bellve & Zheng, 1989), SPARC (43kDa; Vernon & Sage, 1989), PSG (Richardson *et al.*, 1991), IGF- 1 binding protein (IGF-1 BP; Cailleau *et al.*, 1990) and PDGF (Gnessi *et al.*, 1992). There were no proteins on the gel with similar M_r and pI to IL-1, NGF or IGF-1 BP. It is possible that these proteins are either not produced in culture or are produced in insufficient amounts for visualisation on the gels. Protein 1, labelled on the gels in Figure 8.3., has a comparable M_r to PSG. PSG consists of a heterogeneous group of glycoproteins which are synthesised in large quantities in the placenta. PSG mRNA has also been detected in the rat testis and PSG immunoreactivity has been localised to Leydig cells, as well as late spermatids (Ogilvie *et al.*, 1990). PSG mRNA has also been reported in the human testis (Chan *et al.*, 1988) and PSG protein has been found in human semen (Rosen, 1986). Sequence analysis of PSG cDNAs

has revealed the presence of domains with immunoglobulin-like motifs (Richardson *et al.*, 1991). Its function in the placenta is unknown, though it is postulated to have a role in preventing immune rejection of the fetus (Richardson *et al.*, 1991). Similarly, it is possible that PSG in the testis acts as an immunosuppressive factor, and is one of the factors which allows the testis to function as an immune-privileged site.

Protein 2 (Figure 8.3.) has a similar M_r to SPARC. Vernon & Sage (1989) demonstrated that SPARC was synthesized and secreted by Leydig cells isolated from adult mice. SPARC is a calcium-binding protein and is secreted *in vitro* by endothelial cells which are undergoing migratory and proliferative responses (Sage *et al.*, 1986). It is not clear what role SPARC produced by Leydig cells would have. Vernon & Sage (1989) postulated that SPARC was involved in depolymerisation of cytoskeletal elements allowing closer association between lipid droplets and mitochondria thereby facilitating cholesterol uptake by the mitochondria. However this does not explain why Leydig cells secrete SPARC.

A protein localised to Leydig cells which has been proposed to have a paracrine function is PDGF. This protein exists as a dimer of 2 homologous polypeptides, the A and B chains, each with an M_r of about 30kDa. Three forms of PDGF exist, AA, BB and AB (Westermark, 1990). Adult rat Leydig cells produce a PDGF-like activity (Gnessi *et al.*, 1992) and the proteins comprising group 3 (Figure 8.3.) have a comparable M_r to PDGF. PDGF has been shown to act synergistically with transforming growth factor-β to stimulate contraction of rat testicular peritubular cells in culture (Tung & Fritz, 1991).

Figure 8.4. shows the presence of a protein (protein 4) on days 3 and 14 after administration of MAA that is not present on day 7 post-MAA. While protein 4 is not secreted by Leydig cells isolated from control animals and cultured in the absence of hCG, it is secreted by Leydig cells isolated from control animals and cultured in the presence of hCG (Figure 8.3). As it is at days 3 and 14 but not day 7 post-MAA that *in vitro* testosterone production is raised comparative to controls, it appears that the synthesis of protein 4 is associated with increased testosterone production by Leydig cells. The presence of this protein cannot be associated with increased LH(hCG)-stimulation, as serum LH levels are

not significantly different from control levels at 3 and 14 days after MAA administration (Allenby *et al.*, 1991).

The proteins secreted by rat and human Leydig cells are compared in Figure 8.5. There are nine major proteins or protein groups with comparable M_r and pI. These include the proteins tentatively identified in the rat as SPARC, PSG and PDGF. However it appears that while all 3 forms of PDGF may be present in the rat testis, only 2 are produced by human Leydig cells.

In conclusion rat and human Leydig cells have been shown to secrete over 70 proteins. In the rat the influence of the seminiferous epithelium on Leydig cell function does not seem to extend to protein secretion. While culturing Leydig cells in the presence of hCG does not greatly affect protein secretion, conditions of high testosterone production appear to be associated with the production of a novel 35kDa basic protein. Finally, the proteins secreted by rat and human Leydig cells *in vitro* were found to be remarkably similar.

9. *in vitro* interactions between seminiferous tubules and Leydig cells

Chapter 7 described the influence of germ cells, probably acting through Sertoli cells, on Leydig cell function. Depletion of pachytene spermatocytes and/or round spermatids was shown to result in an indirect inhibitory effect on Leydig cell testosterone production, however, only the presence/absence of these germ cells from some spermatogenic stages appeared to have this effect. Specifically, it was suggested that the absence of either pachytene spermatocytes or round spermatids from stage I tubules led to an increase in testosterone production. In chapter 8, the effect of pachytene spermatocytes and/or round spermatids on Leydig cell protein production was assessed and it was found that in situations of high testosterone output *in vitro*, such as the presence of hCG, or the absence of pachytene spermatocytes and/or round spermatids *in vivo*, a protein was secreted that did not appear under basal testosterone conditions. While this protein might have a role in negative feedback to the tubules, these experiments did not shed any light on the mechanism by which germ cells can affect testosterone production. In this chapter staged seminiferous tubules have been used both in co-cultures and to produce conditioned medium, to further examine the nature of the interaction between Leydig cells and the seminiferous epithelium. Testosterone metabolism in the absence and presence of pachytene spermatocytes was measured to see whether these cells act by influencing the rate of testosterone metabolism.

9.1. Introduction

Testicular function is predominantly under the control of pituitary hormones, yet, as has been discussed previously, different cell types in the testis have been shown to influence the function of other testicular cell types, probably by specific secretions. For example, Sertoli cells undergo cyclic changes as the surrounding germ cell population matures. The secretion of many Sertoli cell products is stage-dependent, and frequently

these fluctuations correlate with specific events of spermatogenesis (Parvinen, 1982). The influence of one cell type on another appears to extend beyond the seminiferous epithelium and into the interstitium, as the size of peritubular Leydig cells relates to the stage of the adjacent tubules, with the largest Leydig cells being found around stage VII and VIII tubules (Bergh, 1983). The results presented in chapter 7 also indicate an inter-compartmental interaction, with germ cells apparently able to inhibit the secretion of Sertoli cell factors which stimulate Leydig cell function.

Sertoli cell conditioned medium and Sertoli/Leydig cell co-cultures have been used as experimental models to investigate cross-compartment communication. Sertoli cells isolated from immature (Verhoeven & Cailleau, 1985, Janecki *et al.*, 1985, Grootenhuis *et al.*, 1990) and also adult (Papadopoulos *et al.*, 1987b) rats produce a factor(s) which is able to stimulate testosterone production by rat Leydig cells. The activity is contained within the 10-30kDa M_r range (Verhoeven & Cailleau, 1986), and its secretion appears to be positively regulated by FSH in immature Sertoli cells (Verhoeven & Cailleau, 1986, Janecki *et al.*, 1985) but not in adult Sertoli cells (Papadopoulos *et al.*, 1987b).

Interstitial fluid (IF) which surrounds Leydig cells and seminiferous tubules is the medium through which Leydig cells and Sertoli cells must communicate. Sharpe & Cooper (1984) demonstrated that IF contained a factor(s) which could stimulate testosterone production by cultured Leydig cells. It is not known if this factor corresponds to the 10-30kDa activity described above. Sharpe & Cooper (1984) also showed that IF isolated from germ cell depleted cryptorchid testes had a higher stimulatory effect than control IF. While this points to Sertoli cells as the source of the IF factor, it also suggests that germ cells negatively modulate the secretion of the Sertoli cell factor. A similar interpretation can be made in the study by Papadopoulos *et al.* (1987b), in which seminiferous tubule conditioned medium from irradiated rat testes had greater stimulatory activity than conditioned medium from control rats.

Again, this is in agreement with the experiments described in chapter 7 which demonstrated how depleting seminiferous tubules of pachytene spermatocytes (PS) or round spermatids (RS) *in vivo* led to

increased testosterone production *in vitro*, that is, that PS and RS can negatively modulate the secretion of stimulatory factors from the seminiferous epithelium. However, the effects caused by the absence of PS and RS were not always apparent, in fact, they appeared to be stage-dependent.

The experiments presented in the following chapter have either used co-culture of Leydig cells and seminiferous tubules, or else the addition of seminiferous tubule conditioned medium (STM) to Leydig cells in order to examine the nature of the communication between Leydig cells and the seminiferous epithelium (stimulatory or inhibitory). In order to assess how this interaction changes with the stage of the spermatogenic cycle, as suggested by the experiments described above, all co-cultures used staged seminiferous tubules, and STM was also prepared from staged tubules. As the possibility that germ cells can influence Sertoli cell function has been raised by many authors (see Sharpe, 1992 for review), in some experiments PS were depleted from the seminiferous epithelium using the germ cell toxicant, MAA. The PS-depleted tubules were isolated and staged and cultured with Leydig cells isolated from untreated rats. In this way, the influence of PS on Sertoli cell secretions in tubules of grouped stages was examined. One way in which germ cells could influence the intra-tubular concentration of testosterone is by controlling the rate at which Sertoli cells metabolise testosterone. Thus an increase in testosterone concentrations in the absence of PS would suggest a slower rate of metabolism by Sertoli cells. In order to assess whether isolated seminiferous tubules metabolised significant amounts of testosterone during their co-culture with Leydig cells, staged tubules were incubated alone, and a known amount of testosterone was added to the wells at the start of the culture period. The difference between this value and the amount of testosterone remaining in the medium at the end of the culture period was considered to represent metabolism. Tubules prepared from MAA-treated animals were used to assess the influence, if any, of PS on the rate of metabolism of testosterone.

9.2. Experimental Procedures

9.2.1. Isolation of rat seminiferous tubules

The dissection of seminiferous tubules for co-cultures and for the preparation of seminiferous tubule conditioned medium (STM) was performed by Dr Richard Sharpe.

The testes from a single animal were placed in a glass scintillation vial which was kept on ice until the dissection was performed. When required a testis was decapsulated and placed in a petri dish containing ice-cold Dulbecco's phosphate buffered saline (I.C.N. Flow). This was kept on a trans-illuminated stage, through which ice-cold water was pumped continuously, and which was mounted on a Leitz dissecting microscope. Watchmakers forceps were used to gently tease apart 2-5cm lengths of tubule. These lengths were transferred to fresh phosphate buffered saline. Using the criteria described by Parvinen (1982) the tubules were cut into segments at stages II-V, VI-VIII and IX-I. These segments were never less than 0.5cm in length. In some experiments tubules were dissected from animals that had been treated with MAA 3 days earlier. The preparation and administration of MAA is described in the preceding chapter.

9.2.2. Preparation of staged rat STM

Tubules were dissected as described above and a total of 11.25cm (3.75cm/100µl) of tubules per stage were transferred to separate wells of a 24-well tissue culture plate containing 300µl medium 199 with Earle's salts (M199E) supplemented with 4mM L-glutamine, 0.5mg/ml BSA, 100U/ml penicillin and 100µg/ml streptomycin. The tubules were cultured for 24h at 32°C under a humidified atmosphere of 5% CO_2:95% air. After incubation STM was aspirated carefully and centrifuged at 1000*g* for 5mins to sediment any cellular debris. The supernatant was stored at -20°C until required.

9.2.3. Preparation of unstaged rat STM

Tubules were dissected as described above, but were not staged. Equivalent lengths of unstaged tubules were cultured under the conditions described above, and STM was collected in the same way.

9.2.4. Effect of staged and unstaged rat STM on rat Leydig cell cultures

Staged and unstaged STM were tested for their ability to alter basal and hCG-stimulated testosterone production by Leydig cells *in vitro*. Leydig cells were prepared from control animals as described previously. Following a 2h pre-incubation period, 50,000 Leydig cells were cultured for a further 20h in the presence of 100µl STM in a total incubation volume of 300µl, the volume being made up with M199E. The culture medium was then collected to measure testosterone production by RIA. The amount of testosterone in the STM before its addition to the cultures was also measured.

9.2.5. Co-culture of rat Leydig cells and seminiferous tubules

3.5cm lengths of staged seminiferous tubules were prepared from both control and MAA treated animals. The tubules were added to wells containing 50,000 Leydig cells in 300µl supplemented M199E. Morphometry has shown that in the rat testis there are approximately 200cm seminiferous tubules per $3x10^6$ Leydig cells (Wing & Christensen, 1982). The ratio of Leydig cells to seminiferous tubules used in these co-cultures was therefore within the physiological range. The effect of the presence of seminiferous tubules at stages II-V,VI-VIII or IX-I from control or MAA-treated rats on basal and hCG-stimulated testosterone production by Leydig cells during a 20h culture period was measured. The amount of testosterone in wells containing only seminiferous tubules was also measured.

9.2.6. Assessment of testosterone metabolism by rat seminiferous tubules

The amount of testosterone produced by rat Leydig cells in a 20h culture period was determined to be 138±55ng/10^6 cells (basal conditions, n=41 experiments) and 927±388ng/10^6 cells (hCG-stimulated conditions, n=31). Staged seminiferous tubules, prepared from control and MAA-treated rats were therefore incubated for 20h in supplemented M199E, to which was added either 8µl or 50µl of a solution of 927ng/ml testosterone in M199E (corresponding to basal and hCG-stimulated levels of testosterone. The total incubation volume of 300µl was such that seminiferous tubules were therefore cultured in medium containing a concentration of testosterone that would have been produced over 20h by Leydig cells cultured under these conditions.

9.2.7. Preparation of unstaged human STM

STM was prepared from the testes of a male aged 66 years. The testes were treated according to the protocol described for the preparation of human Leydig cells. That is, the testes were initially subjected to a 50min collagenase digestion, before seminiferous tubule lengths were teased apart with watchmakers forceps. The protocol for incubation was as described for the preparation of unstaged rat STM, except that approximately 50 cm of tubules were incubated in 400µl supplemented M199E for 20h. The total length of tubules was greater than for the rat because of the considerably smaller diameter of human seminiferous tubules.

9.2.8. Effect of unstaged human STM on human Leydig cell cultures

Human STM was tested for its ability to alter basal and hCG-stimulated testosterone production by human Leydig cells *in vitro*. Leydig cells were prepared as described in section 3.3.3. Following a 2h Leydig cell pre-incubation period, Leydig cells were cultured for a further 20h in the presence of 100µl STM in a total incubation volume of 300µl M199E supplemented as above. The amount of testosterone in the STM prior to its addition to Leydig cells was also measured.

9.2.9. Statistics

One-way ANOVA, followed by Students' t-test was used to compare the effects of STM from different stages. Comparisons between the effects of different stages of tubules isolated from either control or MAA treated animals were made using two-way ANOVA followed by Students' t-test.

9.3. Results

9.3.1. The effect of staged rat STM on rat Leydig cell cultures

The effects of STM prepared from tubules at stages II-V, VI-VIII and IX-I on basal and hCG-stimulated testosterone production by rat Leydig cells *in vitro* was assessed in two separate experiments. In one experiment, STM from stages II-V and VI-VIII had no effect on basal testosterone production, while the presence of STM from stages IX-I had a significant stimulatory effect when compared to testosterone production in the absence of STM (p <0.01, Figure 9.1.A). In the second experiment testosterone production was not affected by the presence of STM from any stage (ANOVA p =0.206, Figure 9.1.C). In the first experiment STM from all stages had a significant stimulatory effect on hCG-stimulated testosterone production (p <0.05, Figure 9.1.B). However, in the second experiment, STM from stages II-V and IX-I did not have a significant effect on hCG-stimulated testosterone production, while STM from stages VI-VIII had a significant **inhibitory** effect (p <0.05, Figure 9.1.D).

9.3.2. Co-culture of rat Leydig cells and seminiferous tubules

Testosterone production by Leydig cells co-cultured with seminiferous tubules from either of the three stage groupings was very variable. Only stage II-V tubules which had been isolated from control rats appeared to have any influence on testosterone production by Leydig cells. When compared to the amount of testosterone produced by Leydig cell-only cultures, stage II-V tubules had an inhibitory effect on basal testosterone production (p < 0.05, Figure 9.2A).

At 3 days after administration of MAA, seminiferous tubules at most stages are depleted of 80-100% of pachytene spermatocytes (PS). However, tubules prepared from MAA-treated animals and cultured with Leydig cells, did not produce effects different to those seen with tubules isolated from control animals, except that the inhibitory effect of stages II-V was not evident with tubules prepared from MAA-treated animals (Figure 9.2.A). Figure 9.2B demonstrates that there was no significant effect of tubules at any stage, from control or MAA-treated animals, on hCG-stimulated testosterone production by Leydig cells (ANOVA p =0.978).

9.3.3. Testosterone metabolism by seminiferous tubules

The ability of seminiferous tubules prepared from either control or MAA-treated rats to metabolise a known amount of testosterone *in vitro* was compared in two separate experiments. Seminiferous tubules in all stage groupings metabolised appreciable amounts of testosterone, ranging from 7.4-49% (basal) and 12.5-50.7% (hCG-stimulated) The control values in the graphs comprising Figure 9.3., represent the amount of testosterone added initially to each well. In one group of wells the amount of testosterone added to each well was of an equivalent order to the 20h basal rate of testosterone production by Leydig cells. In the first experiment, the extent of testosterone metabolism by any one group of stages (II-V, VI-VIII, IX-I), was independent of whether the tubules had been prepared from control or MAA-treated animals (Figure 9.3.A). In the second experiment stage IX-I tubules from MAA-treated animals metabolised significantly less testosterone than did tubules from control animals (p <0.01, Figure 9.3.B). While a significantly greater amount of testosterone had been added to tubules prepared from MAA-treated animals than to those prepared from control animals, this alone does not account for the discrepancy between the groups.

When the amount of testosterone added to each well was of an equivalent order to the 20h hCG-stimulated rate of testosterone production by Leydig cells, similar results were found. In both experiments, whether the tubules had been prepared from control or MAA-treated animals did not effect the ability of any particular grouping

of stages to metabolise testosterone (Figure 9.3.C and D). As the absence of PS, caused by *in vivo* treatment with MAA had no consistent significant effect on the activity of seminiferous tubules in the stage groupings used in these experiments, only results from experiments with control tubules will be discussed further. The degree of testosterone metabolism by tubules prepared from control animals in 3 separate experiments is shown in Table 9.1. Although the individual variation in the metabolism of the 'basal' amount of testosterone is high, tubules at stages VI-VIII metabolised a significantly greater amount of testosterone than did tubules at stages II-V or IX-I (p <0.05, Table 9.1.). No significant difference was found in the ability of tubules at any stage grouping to metabolise the 'hCG-stimulated' amount of testosterone (ANOVA p =0.729, Table 9.1).

In Figures 9.4. and 9.5. the amounts of testosterone measured in Leydig cell and seminiferous tubule co-cultures have been 'corrected' by the appropriate factor to take into account the metabolism of testosterone measured in the experiment described previously. The results of two separate experiments are shown. Figure 9.4.A demonstrates how the amount of testosterone metabolised by all stage groupings of control tubules significantly affected the measure of basal testosterone production by Leydig cells. This was also found in the second experiment for all stages considered (Figure 9.4.B). The distorting affect of metabolism by seminiferous tubules on the assessment of hCG-stimulated production of testosterone by Leydig cells in co-culture, was less clear-cut. In one experiment, only testosterone metabolism by stage IX-I tubules significantly affected the measured level of testosterone (Figure 9.4.C), while in the second experiment, while metabolism by stages II-V and VI-VII did significantly affect the measured level of testosterone, stages IX-I did not (Figure 9.4.D).

9.3.4. The effect of unstaged rat STM on rat Leydig cell cultures and of unstaged human STM on human Leydig cell cultures

Unstaged rat STM had no effect on basal testosterone production by rat Leydig cells during culture for either 4 or 20h (Figure 9.5.A). The effect of STM on hCG-stimulated testosterone production was inhibitory at both time points (p <0.01, Figure 9.5.A).

Figure 9.1. The effect of staged rat STM on testosterone production by rat Leydig cells cultured for 20h.

A. basal testosterone production in a single experiment. **B.** hCG-stimulated testosterone production in the same experiment. **C.** basal testosterone production in a second experiment. **D.** hCG-stimulated testosterone production in the second experiment. Each point is the mean±S.D. of triplicate wells. $*p < 0.05$, $**p < 0.01$ significantly different from control values.

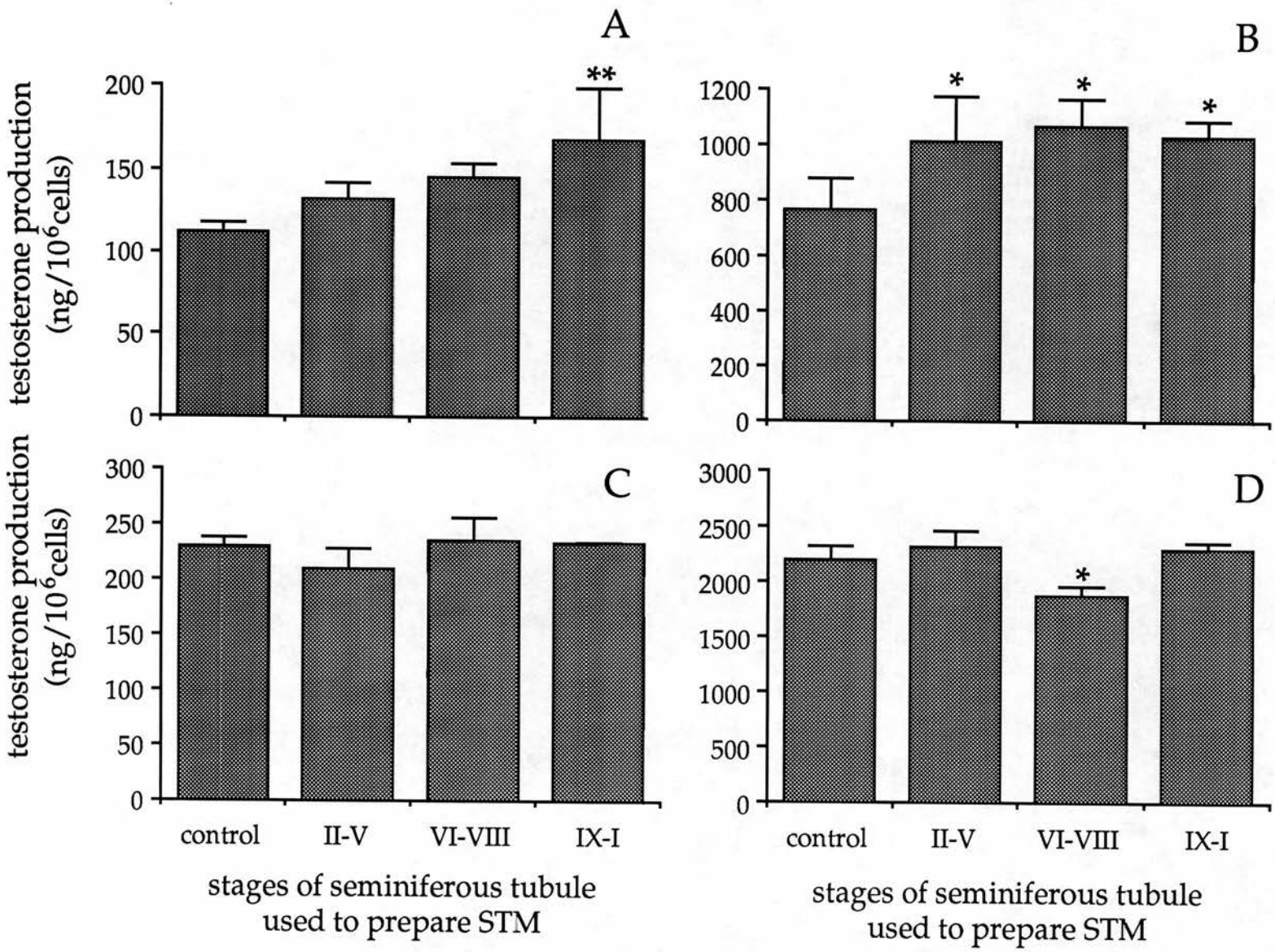

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
200
150
100
50
0
1200
1000
800
600
400
200
0
300
250
200
150
100
50
0
3000
2500
2000
1500
1000
500
0
**
*
*
*
*
control
II-V
VI-VIII
IX-I
control
II-V
VI-VIII
IX-I
stages of seminiferous tubule used to prepare STM
stages of seminiferous tubule used to prepare STM

Figure 9.2. 20h co-culture of rat Leydig cells with control and germ cell depleted seminiferous tubules - effect on testosterone production.

A. basal testosterone production by rat Leydig cells (n=5 for co-cultures with control seminiferous tubules, n=3 for co-cultures with germ cell depleted seminiferous tubules). **B.** hCG-stimulated testosterone production by rat Leydig cells (n=5 for co-cultures with control seminiferous tubules, n=3 for co-cultures with germ cell depleted seminiferous tubules). Each point is the mean±S.D. of n experiments. *p <0.05 significantly different from control values.

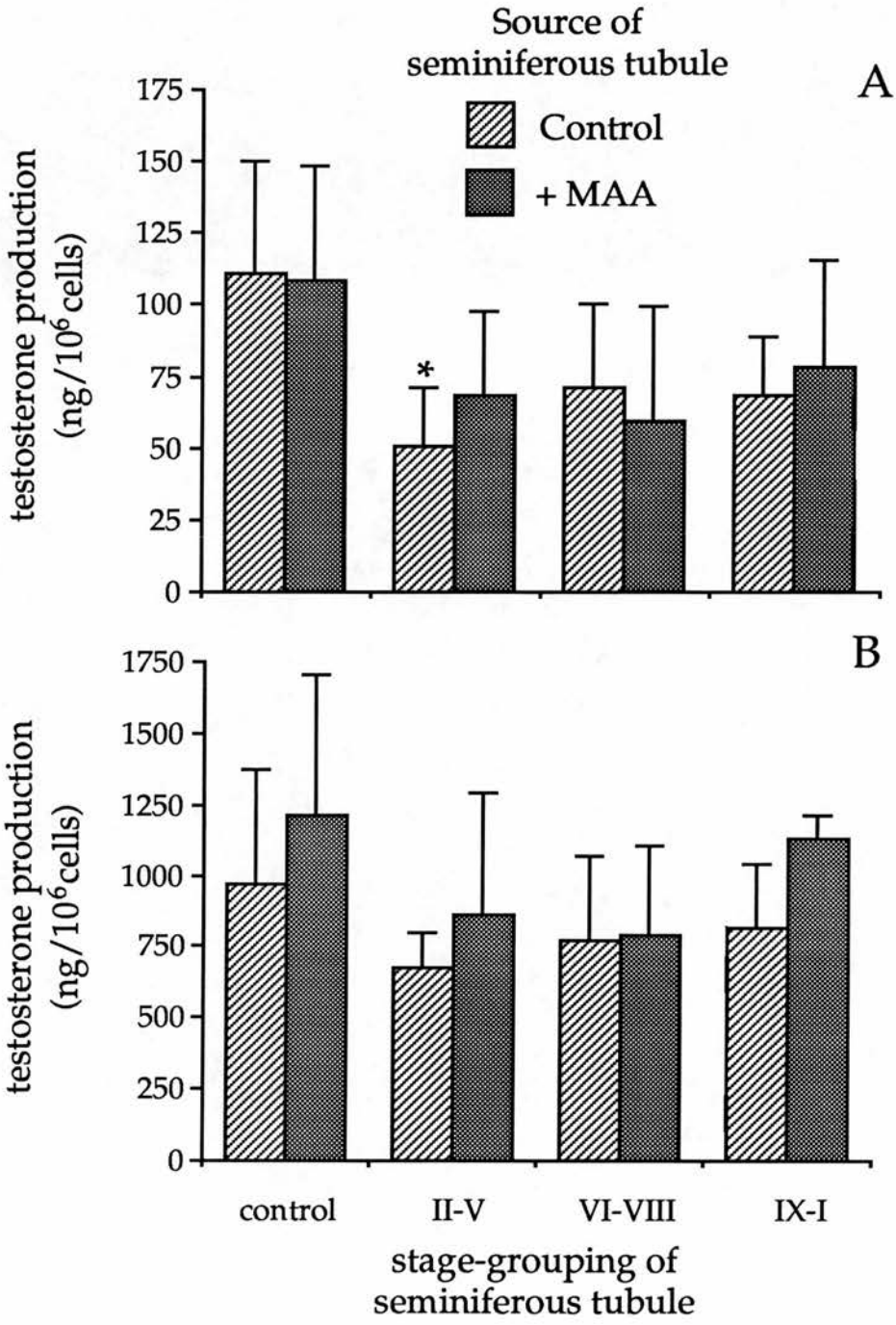

Source of
seminiferous tubule
A
Control
+ MAA
testosterone production
(ng/10^6 cells)
175
150
125
100
75
50
25
0
*
B
1750
1500
1250
1000
750
500
250
0
control
II-V
VI-VIII
IX-I
stage-grouping of
seminiferous tubule

Figure 9.3. Testosterone metabolism by control and germ cell depleted seminiferous tubules cultured for 20h.

A. metabolism of 'basal' testosterone levels in a single experiment. **B.** metabolism of 'basal' testosterone levels in a second experiment. **C.** metabolism of 'hCG-stimulated' testosterone levels in the first experiment. **D.** metabolism of 'hCG-stimulated' testosterone levels in the second experiment. Each point is the mean±S.D. of triplicate wells. $*p < 0.05$, $**p < 0.01$ level in germ cell depleted tubules significantly different from levels in control tubules at the same stage.

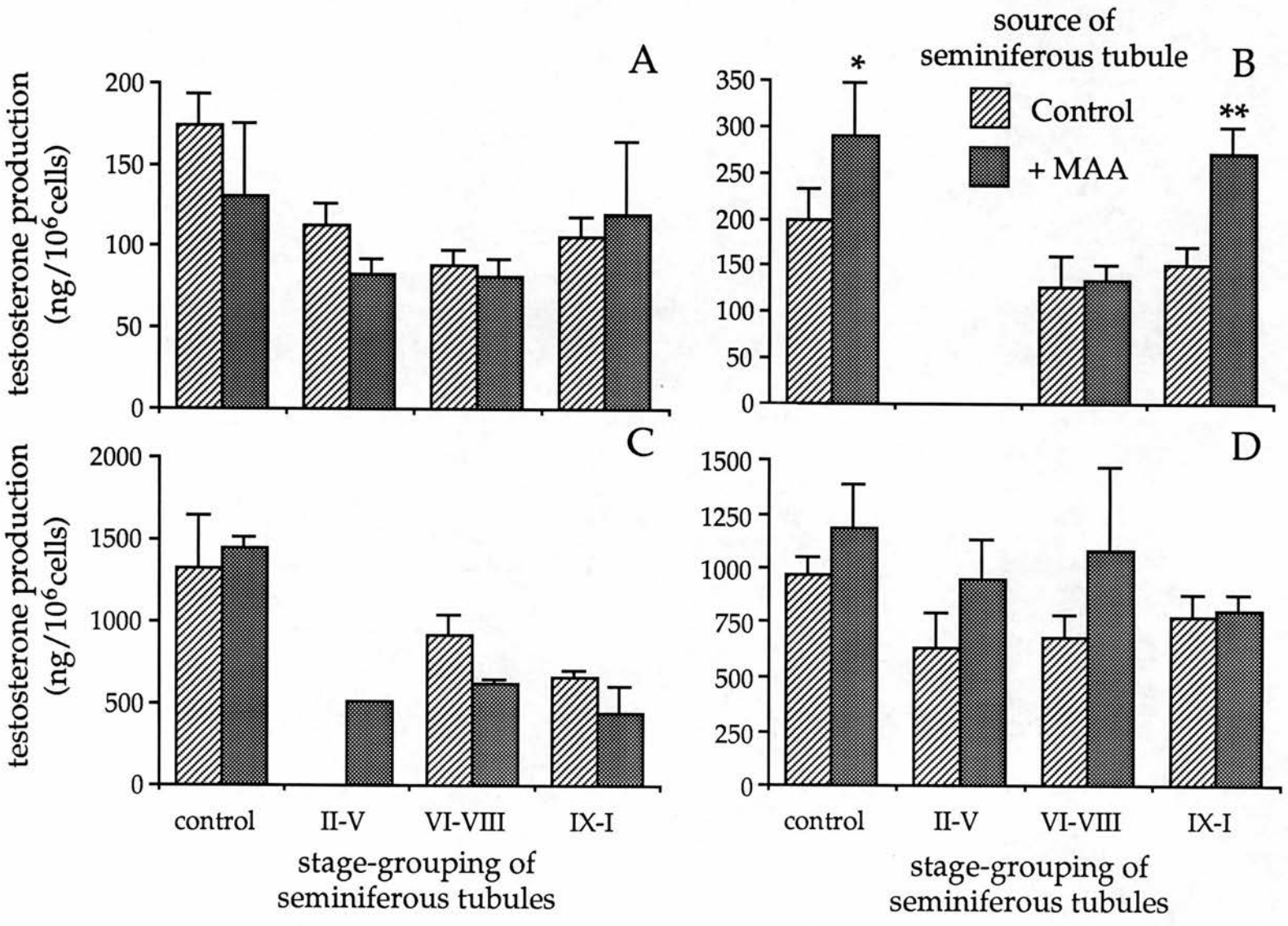

A
B
C
D
testosterone production (ng/10^6 cells)
testosterone production (ng/10^6 cells)
source of seminiferous tubule
Control
+ MAA
*
**
control
II-V
VI-VIII
IX-I
stage-grouping of seminiferous tubules
stage-grouping of seminiferous tubules

Table 9.1. The extent of testosterone metabolism by seminiferous tubules cultured for 20h.

The results from 3 experiments and the mean values of those experiments are shown. * $p < 0.05$ significantly different from other stages.

percentage of 'basal' testosterone metabolised by seminiferous tubules

stage	II-V	VI-VIII	IX-I
expt.no			
1	7.4	18.2	10.9
2	34.9	49.0	36.3
3	32.4	37.9	26.9
average±SD	24.9±15.2	35.03±15.6*	24.7±12.8

percentage of 'hCG-stimulated' testosterone metabolised by seminiferous tubules

stage	II-V	VI-VIII	IX-I
expt.no			
1	12.5	18.4	26.4
2	40.4	30.1	50.7
3	35.6	29.9	20.6
average±SD	29.5±14.9	26.1±6.7	32.6±15.9

Figure 9.4. The effect of correcting for testosterone metabolism by seminiferous tubules on the levels of testosterone measured in culture medium in 20h Leydig cell/seminiferous tubule co-cultures.

A. uncorrected and corrected measurements of basal testosterone production in a single experiment. **B.** uncorrected and corrected measurements of basal testosterone production in a second experiment. **C.** uncorrected and corrected measurements of hCG-stimulated testosterone production in the first experiment. **D.** uncorrected and corrected measurements of hCG-stimulated testosterone production in the second experiment. Each point is the mean±S.D. of triplicate wells. $*p < 0.05$, $**p < 0.01$ corrected value significantly different from uncorrected value at the same stage. $+p < 0.05$, $++p < 0.01$ significantly different from uncorrected control values.

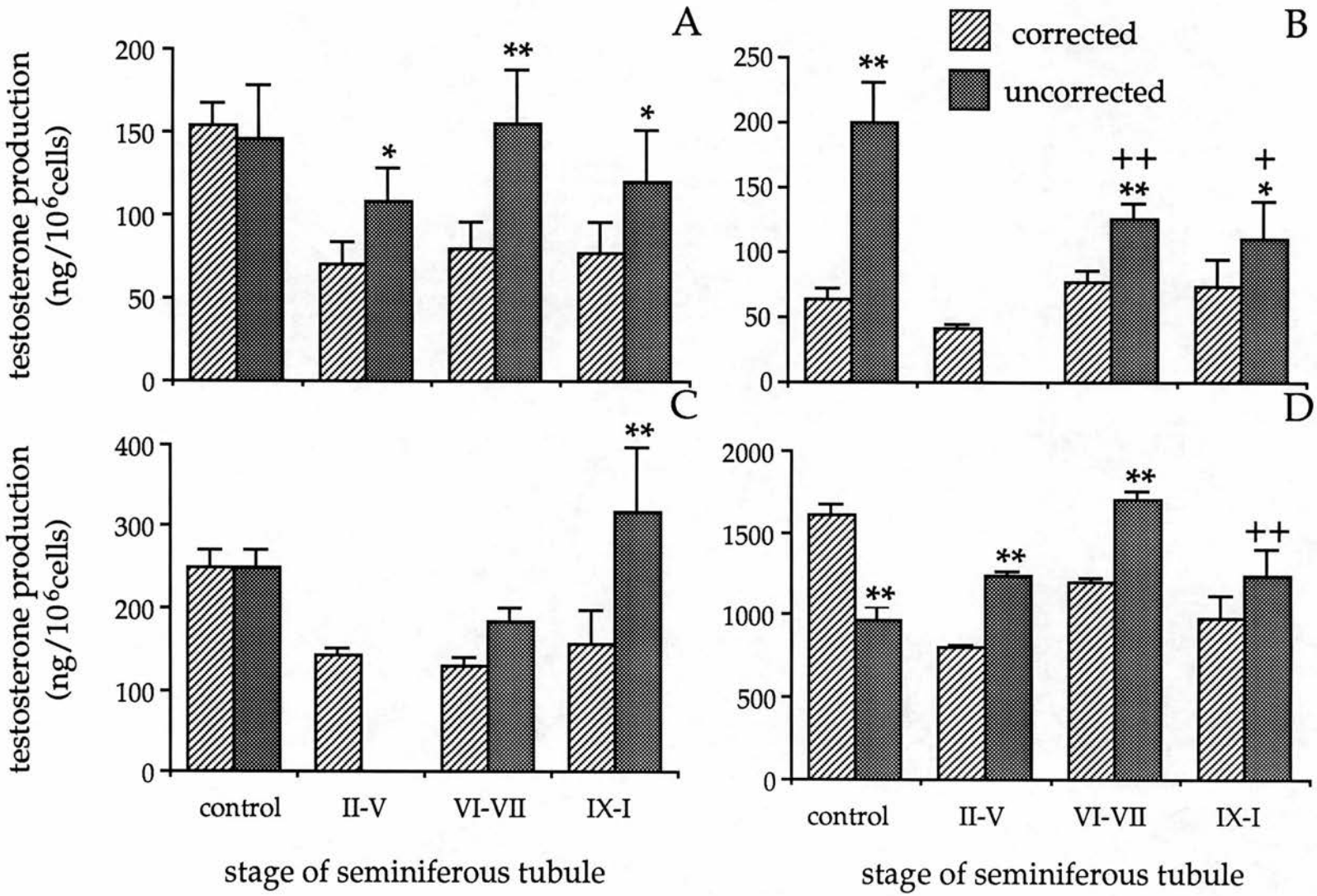

A
B
C
D
corrected
uncorrected
testosterone production
(ng/10^6cells)
testosterone production
(ng/10^6cells)
control
II-V
VI-VII
IX-I
control
II-V
VI-VII
IX-I
stage of seminiferous tubule
stage of seminiferous tubule

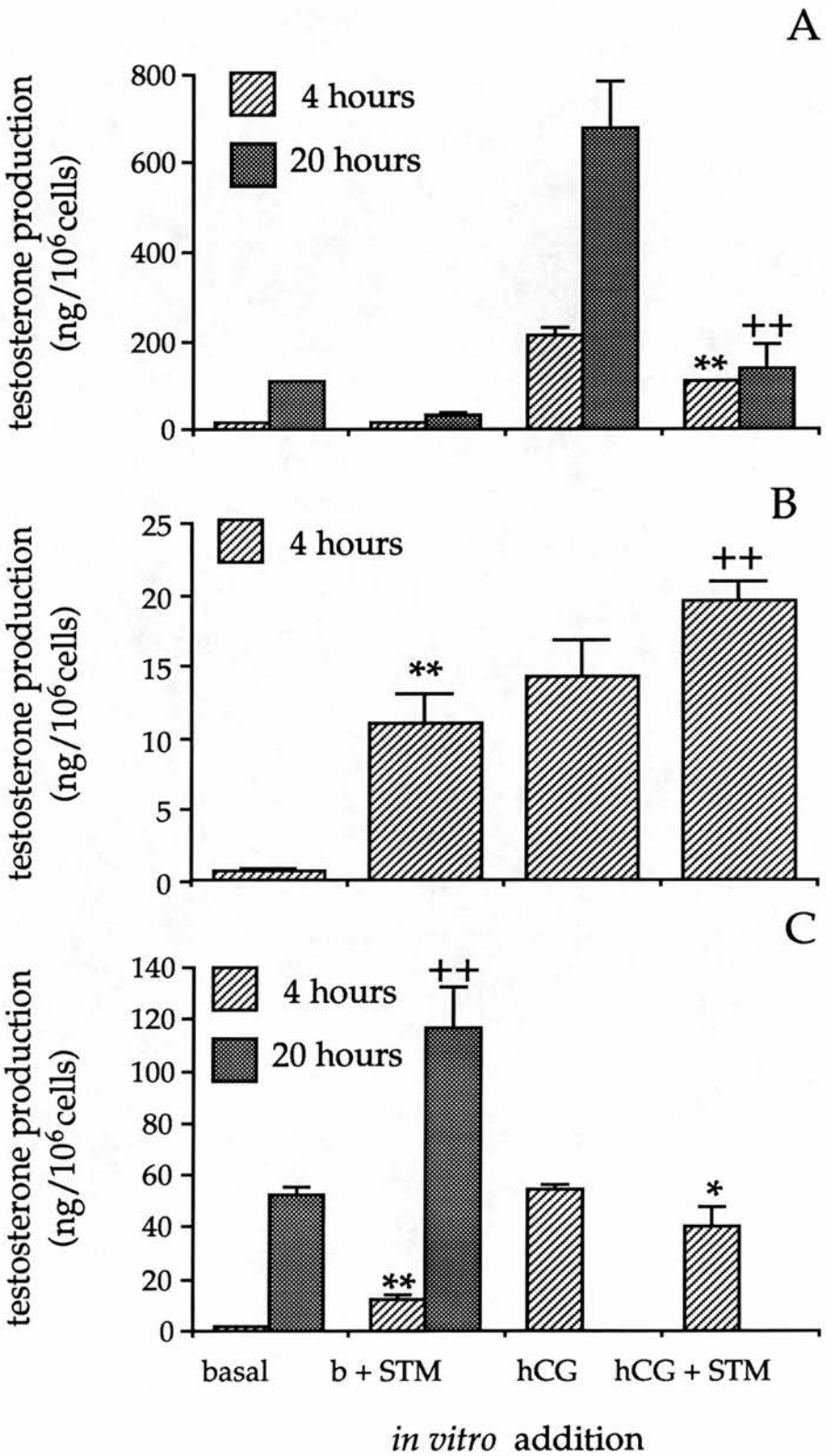
Figure 9.5. The effect of unstaged rat and human STM on testosterone production by rat and human Leydig cells respectively, each cultured for 4 and 20h.

A. testosterone production by rat Leydig cells in one experiment.

B. testosterone production by human Leydig cells in a single experiment.

C. testosterone production by human Leydig cells in a second experiment.

Each point is the mean±S.D. of triplicate wells. $*p < 0.05$, $**p < 0.01$ significantly different from control values.


A
testosterone production (ng/10^6cells)
800
600
400
200
0
4 hours
20 hours
**
++
B
testosterone production (ng/10^6cells)
25
20
15
10
5
0
4 hours
**
++
C
testosterone production (ng/10^6cells)
140
120
100
80
60
40
20
0
4 hours
20 hours
**
++
*
basal
b + STM
hCG
hCG + STM
in vitro addition

The effect of human STM prepared from a man aged 66 years, was tested on 2 occasions. In both experiments STM had a stimulatory effect on basal testosterone production at 4h (p <0.01, Figures 9.5.B and C), and in one experiment the stimulatory effect was also seen at 20h (p <0.05, Figure 9.5.C). The effect of human STM on hCG-stimulated testosterone production was variable. In the first experiment STM had a stimulatory effect on hCG-stimulated testosterone production (p <0.01, Figure 9.5.B), while in the second STM was found to have an inhibitory effect on hCG-stimulated testosterone production (p <0.01, Figure 9.5.C).

9.4. Discussion

The aims of the experiments described in this chapter were to use Leydig cell/seminiferous tubule co-cultures as a model system for examining cell-cell interactions in the rat and human testis. It was hoped that by using the germ cell specific toxin, MAA, to deplete the seminiferous epithelium of PS and then co-culturing Leydig cells with such germ cell depleted seminiferous tubules, it would be possible to assess the contribution that germ cells make to Leydig cell regulation. In chapter 7 it was postulated that it is seminiferous tubules at stage I which secrete a factor(s) with an inhibitory action on Leydig cell steroidogenesis, thus an additional aim of these experiments was to use staged seminiferous tubules from MAA-treated rats to examine this hypothesis further.

Locally produced paracrine factors are believed to contribute to the coordinated action of the interstitial compartment and the seminiferous epithelium. For example, during testicular development FSH increases the capacity of Leydig cells to produce testosterone in response to LH stimulation (Odell & Swerdloff, 1976) and promotes Leydig cell maturation (Kerr & Sharpe, 1985), yet receptors for FSH are found only in the tubular compartment. More recent and more direct evidence of interactions between these testicular compartments has already been detailed in chapter 7. In general, most authors have taken one of two approaches to investigate cell-cell interactions in the testis. One method is to co-culture Leydig cells with Sertoli cells, or Leydig cells with seminiferous tubules and examine the effect on testosterone production. The alternative approach is to analyse the effect of spent medium from

either Sertoli cells or seminiferous tubules on Leydig cell testosterone production.

Two separate experiments assessed the effects of medium conditioned by seminiferous tubules at stages II-V, VI-VIII or IX-I on Leydig cell function. In one experiment the addition of STM from any of these stage groupings had no effect on basal testosterone production, while in the second experiment STM from stages IX-I stimulated basal testosterone production. The effect of STM on hCG-stimulated testosterone production was also inconsistent between experiments. In the first, STM from all stages had a significant stimulatory effect on hCG-stimulated testosterone production, while in the second, STM from stages II-V or IX-I had no effect and STM from stages VI-VIII was inhibitory. While these results are inconclusive they do serve to illustrate the confusion that also exists in the literature regarding the effects of conditioned media on Leydig cell function. Seminiferous tubule conditioned media has previously been shown to have stimulatory effects on testosterone production (Papadopoulos *et al.*, 1987b, Bartlett *et al.*, 1987), inhibitory effects (Syed *et al.*, 1988, Vihko & Huhtaniemi, 1989) and also to have no effect on testosterone production (Parvinen *et al.*, 1984). While these differences may be attributable to differences in methodology, it is not immediately clear what such differences might be. For example, all the studies quoted used adult rats to isolate the Leydig cells and seminiferous tubules, and all used Percoll-purified Leydig cells. The study by Bartlett *et al.* (1987) was the only one to use collagenase digestion in the process of isolating seminiferous tubules, yet this cannot contribute to the differences in effects seen as Bartlett *et al.* (1987) observed the same stimulatory effects as Papadopoulos *et al.* (1987b) who used the dissection technique described by Parvinen & Vanha-Perttula (1972) to isolate seminiferous tubules. Neither does the method of co-incubation appear to influence the effect of STM on Leydig cells as inhibitory effects have been recorded using monolayer cultures (Syed *et al.*, 1988) and incubations in a shaking water bath (Vihko & Huhtaniemi, 1989). The results presented in this chapter which show that overall STM does not have a consistent effect on basal or hCG-stimulated testosterone production, are most in agreement with those of Parvinen *et al.* (1984) showing no effect of STM on Leydig cell function. These authors

suggested that the lack of effect of STM may be due to a dynamic interaction between Leydig cells and seminiferous tubules being a necessity for cell-cell communication.

To accommodate the possibility that such an interaction is required for communication, a second series of experiments were undertaken using co-culture of Leydig cells and staged seminiferous tubules. Although there was a high degree of variation between experiments, the presence of tubules at stages II-V consistently had an inhibitory effect on basal testosterone production, while the presence of tubules at other stages had no effect. hCG-stimulated testosterone production was not affected by seminiferous tubules from any stage. Papadopoulos *et al.* (1987b) used irradiated rats to show that STM collected from germ cell depleted seminiferous tubules was able to stimulate 17β-oestradiol production more than was STM from control rats. This suggested that germ cells might have a negative influence on the synthesis of a Sertoli cell secreted factor, the activity of which had been described in the same series of experiments. The experiments presented in chapter 7 also suggested that germ cells, specifically PS and RS, were able to negatively influence the production of seminiferous epithelial factors with stimulatory activity on Leydig cell function. To investigate this further, tubules were isolated from rats in which the testes had been depleted of PS by the administration of MAA 3 days previously. As with tubules isolated from control animals the variation between experiments was quite high. The only difference in effect on Leydig cell testosterone production caused by using seminiferous tubules depleted of PS, was that the inhibitory effects of stage II-V tubules were no longer present. In all other aspects there was no difference between the effects of tubules from control or MAA-treated rats. The lack of inhibition of stages II-V after the removal of PS is consistent with the results presented in chapter 7, in which removal of PS corresponded to the removal of an inhibitory influence and increased basal testosterone production above control levels. In these co-culture experiments, the removal of PS also appeared to be associated with the loss of an inhibitory influence and testosterone levels were brought back within the control range. However, the lack of effect of seminiferous tubules isolated from control animals at stages VI-VIII and IX-I does not agree with previously published studies. As with

the effects of STM the reported effects of co-cultures vary. Parvinen *et al.* (1984) reported inhibitory effects of all stages on both basal and hCG-stimulated testosterone production by crude preparations of Leydig cells, although when using Percoll-purified Leydig cells all stages had stimulatory effects on both basal and hCG-stimulated testosterone production. Bartlett *et al.* (1987) found no effect of any stage on either basal or hCG-stimulated testosterone production. Again it appears that the methodology used has a great influence on the results obtained. In the experiments presented in this chapter the minimum length of seminiferous tubule used was 0.5cm. In the study by Parvinen *et al.* (1984) seminiferous tubule were cut into lengths of only 2mm. It has previously been suggested that leakage from seminiferous tubules is via tubular fluid escaping from the cut ends of the tubules rather than across the Sertoli cells and basement membrane (Sharpe & Bartlett, 1985). Thus using many small pieces of tubule will result in a greater diffusion of tubular fluid than using fewer large pieces. Leydig cells thus become exposed to Sertoli or germ cell products that are normally sequestered in tubular fluid. The significance of this was indicated in a study by Onoda *et al.* (1991), who showed that PS proteins added to Leydig cell cultures had a direct stimulatory effect on testosterone production. The action of such a protein(s) to which Leydig cells are not normally exposed *in vivo* could explain the stimulatory effects seen by Parvinen *et al.* (1984).

Based on the results of a morphometric study, Bergh (1983) proposed the existence of a stage-dependent paracrine regulation of Leydig cells by adjacent Sertoli cells. Evidence suggested that peritubular Leydig cells surrounding stage VII-VIII tubules were larger than peritubular Leydig cells surrounding any other stage. This is in agreement with the proposal that testosterone acts only at stage VII (Russell & Clermont, 1977), and synthesis of ABP is also highest at stages VII-VIII (Ritzen *et al.*, 1982). However, Parvinen & Huhtaniemi (1990) have shown that per unit volume the concentration of testosterone in freshly isolated dry-dissected seminiferous tubules is identical in all stages of the epithelial cycle. To reconcile this with the apparent differential in testosterone action at different stages of the spermatogenic cycle an experiment was undertaken to investigate the extent to which tubules at different stages metabolised testosterone. Using tubules generated from MAA-treated animals and

therefore depleted of PS, the effect of PS on testosterone metabolism was also assessed. Syed *et al.* (1985) have shown that there are no factors in seminiferous tubule conditioned medium that can increase the metabolism of testosterone. Parvinen *et al.* (1984) demonstrated that *in vitro* seminiferous tubules themselves cannot convert Leydig cell derived precursors to testosterone. The experiments presented in this chapter show that while tubules at all stages can metabolise testosterone, it is greatest at stages VI-VIII. This is in agreement with these stages being testosterone-dependent. The depletion of PS from the tubules had no effect on the metabolism of testosterone except in one experiment, in which the absence of PS led to a significantly smaller degree of testosterone metabolism by tubules depleted of PS when compared to controls. Less metabolism would presumably result in higher testosterone levels, again indicating that PS in early stages have an inhibitory effect on local testosterone concentrations.

If the amount of testosterone produced by Leydig cells in co-culture is 'corrected' to allow for metabolism by the tubules, the corrected testosterone level is higher than the measured testosterone level. The effect seminiferous tubules at different stages on Leydig cell testosterone production also changes. When no allowance was made for testosterone metabolism, seminiferous tubules at stages II-V had an inhibitory effect on basal testosterone production while seminiferous tubules at stages VI-VIII and IX-I had no effect. After correcting for metabolism, seminiferous tubules at all stages had no effect on testosterone levels in one experiment, while in the second experiment, the effect of seminiferous tubules at stages VI-VIII and IX-I were stimulatory while seminiferous tubules at stages II-V had no effect. After correcting for the effects of metabolism on hCG-stimulated testosterone production, the presence of seminiferous tubules at stages II-V and VI-VIII had no effect on hCG-stimulated Leydig cell testosterone production in either experiment, while seminiferous tubules at stages IX-I had no effect in one experiment, but were inhibitory in the other. Before these corrections were made no stage had any effect on testosterone production. Without reproducible results it is not possible to speculate on the interactions that occur between Leydig cells and the seminiferous epithelium and how such interactions might change with the stage of the cycle. However it is possible to say that in co-cultures of

Leydig cells and seminiferous tubules, the tubules metabolise a significant amount of testosterone, and the degree of metabolism is greatest at stages VI-VIII. The idea that the testosterone produced by Leydig cells *in vitro* is metabolised by seminiferous tubules during co-culture has received little, if any, attention previously. Although no attempt was made to measure the products of testosterone metabolism, one of the major metabolites is dihydrotestosterone (DHT), which is known to have a greater affinity for the androgen receptor than testosterone itself (Rommerts, 1989, for review). Metabolism to DHT would therefore effectively increase androgen levels. As the highest degree of testosterone metabolism was by seminiferous tubules at stages VI-VII, the subsequent maximal availability of DHT would be consistent with the androgen-dependent nature of these stages (Sharpe, 1992).*

As human seminiferous tubules cannot be dissected into distinct stages in the way that rat tubules can, any *in vitro* study of human Leydig cell/seminiferous tubule interactions must use unstaged seminiferous tubules. In a single comparative experiment unstaged rat STM was found to have no effect on basal testosterone production by Leydig cells at either 4 or 20h. The effect on hCG-stimulated testosterone levels was inhibitory at both time points. In contrast, human STM stimulated basal testosterone production by human Leydig cells at both 4 and 20h in two separate experiments. However, in one experiment STM had an inhibitory action on hCG-stimulated testosterone production, while in the second its effect was stimulatory. It is interesting to note that the human STM was prepared from the testes of a man aged 66 years, whose Leydig cells produced extremely low amounts of testosterone *in vitro*; 0.5ng/10^6cells/20h basal compared with the average of 69.45ng/10^6cells/20h. If seminiferous tubule factors can influence Leydig cell testosterone output, then it is possible that tubules from testes in which the Leydig cells have a very low basal testosterone secretion level, might produce high amounts of a steroidogenesis-stimulating factor(s). Thus the Leydig cells isolated from the testes from which the STM was prepared were unable to produce much testosterone *in vitro* due to the absence of this factor.

It is also possible that Leydig cells which are low producers of testosterone might be more responsive to such a tubular factor than

* Equivalent measurements of testosterone levels before and after charcoal extraction revealed that the discrepancy in testosterone levels in these experiments was not due to binding to ABP.

Leydig cells which were producing testosterone in sufficient amounts to maintain spermatogenesis. This is supported by the finding that the human Leydig cell preparation which was most responsive to the effects of STM was a lower producer of testosterone *in vitro* than the preparation which was less responsive to STM (basal testosterone was 9.4ng/10^6cells/20h compared with 53.19ng/10^6cells/20h). This might also explain the discrepancy between the reported effects of rat and human STM, as rat STM was prepared from normally fertile animals, with no problems associated with ageing and all the other reproductive advantages of their species.

Verhoeven & Cailleau (1987) demonstrated that conditioned medium from cultured human seminiferous tubule fragments was able to stimulate testosterone output from a crude preparation of human interstitial cells. Characterisation of the active factor showed it to have an M_r in the range 10-30kDa. Verhoeven & Cailleau (1986) had previously shown that rat Sertoli cells produce a factor(s) with a similar M_r which stimulates rat Leydig cell activity. In fact conditioned media from rat and human seminiferous tubule cultures were found to have parallel dose-response curves. The activities of the rat and human factors were both increased by *in vitro* treatment with FSH, and the authors postulated that rat and human Sertoli cells produce an identical factor the function of which is to mediate the effects of FSH on Leydig cell function. Papadopoulos *et al.* (1991) found that human Sertoli cell cultures produce a 79kDa protein with a stimulatory effect on Leydig cell function. It is not known whether either of these factors are responsible for the actions of human STM described in this study.

In summary, it was not possible to clearly define the influence of seminiferous tubules from each stage of the spermatogenic cycle on Leydig cell testosterone production. However, co-culture experiments indicated that cells in the early stages of the cycle (II-V) have an inhibitory effect on testosterone production. In co-cultures in which PS had been depleted from seminiferous tubules at stages II-V, this inhibitory effect was lost, suggesting the involvement of PS in the production of an inhibitory factor(s). In an earlier chapter it was suggested that PS at stage I had an important negative modulation on Sertoli cell function. The discrepancy reported here, in which PS at stages II-V are the important

cells could either be because it has not been possible to dissect stage I from stage II, though it is more likely to mean that PS at all of the *early* stages of the spermatogenic cycle exert a negative effect on Sertoli cell modulation of Leydig cell function..

It was also shown that a significant amount of testosterone was metabolised by tubules at each stage. The absence of PS from tubules did not affect testosterone metabolism, thus this is not the mechanism by which PS alter testosterone levels *in vitro*. In accordance with stages VI and VIII being the major sites of testosterone action and also the stages at which ABP secretion is highest, the greatest degree of testosterone metabolism occurred at stages VI-VIII.

10. Toxicology of rat and human Leydig cells

Preceding chapters have described many aspects of Leydig cell function, especially with regard to potential intercellular control mechanisms. Where possible comparisons were made between rat and human Leydig cell function, and many similarities were found. This chapter examines the susceptibility of Leydig cells from both species to the adverse effects of 4 compounds, and shows how the similarity between rat and human Leydig cells makes rat Leydig cells useful models in toxicological hazard evaluations.

10.1. Introduction

Rat Leydig cells have been used extensively as convenient models for human Leydig cells in studies of endocrine and paracrine control of steroidogenesis, and also in toxicological assessments, when the adverse effects of new drugs or chemicals on reproductive function are evaluated (Lamb, 1987). However very little is known about human Leydig cells, and as many functional variations have been demonstrated in other species (Saez *et al.*, 1987, Dufau, 1988), an extrapolation from rat to human cannot be safely assumed. The aim of the experiments described in this chapter was to compare, in rat and human Leydig cells, the effects of 4 compounds with adverse effects on steroidogenesis.

Ketoconazole (KCZ) is an imidazole derivative which was used clinically as an anti-fungal agent. In yeast KCZ inhibits the demethylation of lanosterol to ergosterol, thus disrupting cell membrane formation. The clinical administration of KCZ is associated with gynaecomastia in a small percentage of men. This led to studies of androgen levels after administration and it was found that doses of between 200 and 600 mg KCZ caused a dose-dependent decrease in plasma testosterone levels. This was accompanied by an increase in plasma LH, suggesting that KCZ exerted its effects at the testicular level. KCZ has since been shown to inhibit testosterone production *in vitro* by blocking the action of cytochrome $P_{450}17\alpha$ (Sikka *et al.*, 1985). The effect of KCZ on highly

purified human Leydig cells has not been demonstrated previously. In order to determine whether rat Leydig cells could be used as a guide to the relative efficacies of related compounds on human Leydig cell function, the effects of hexaconazole (HCZ), a triazole anti-fungal agent, were compared in rat and human. Tralkoxydim is a selective herbicide the mechanism of action of which is unknown, and its effects on steroidogenesis in the rat and in man were also compared.

Ethane dimethane sulphonate (EDS) is an alkylating agent, although it is not clear whether this action is related to its *in vivo* effects on rat Leydig cells. Administration of EDS to adult rats results in the destruction of all Leydig cells between 1 and 3 days post-treatment (Kerr *et al.*, 1985, Morris *et al.*, 1986). Inhibitory effects of EDS on steroidogenesis in isolated rat Leydig cells has been described previously (Rommerts *et al.*, 1988), although it was not possible to relate this effect to the destruction of Leydig cells observed *in vivo*.

In order to investigate the validity of cultured rat Leydig cells as a model for human Leydig cell function *in vitro*, the effects of the above compounds on basal and hCG-stimulated testosterone production in rat and human Leydig cells were compared.

10.2. Experimental Procedures

Rat and human Leydig cells were prepared using the methods described in chapter 3. The effects of various *in vitro* treatments on both basal and hCG-stimulated testosterone production was assessed over a 20h culture period. Preparation of the drug solutions used is described below. ATP levels were measured at the end of each culture period to confirm that any effects were not secondary to cell death.

Ketoconazole (KCZ) : Stock solutions were prepared by dissolving KCZ (Sigma) in 1% DMSO. To achieve final culture well concentrations in the range 0.01-100µg/ml (Kan *et al.*, 1985), the stock solution was diluted in medium 199 with Earles' salts (M199E). The final concentration of DMSO in each well was 0.05%v/v.

Hexaconazole (HCZ) : Stock solutions were prepared by dissolving HCZ (ICI, Macclesfield) in 1% DMSO. To achieve final culture well concentrations in the range 0.01-100μg/ml, the stock solution was diluted in M199E. The final concentration of DMSO in each well was 0.05%v/v.

Tralkoxydim (TKD) : Stock solutions were prepared by dissolving TKD (ICI, Macclesfield) in 1% DMSO. To achieve final culture well concentrations in the range 0.1-100μg/ml, the stock solution was diluted in M199E. The final concentration of DMSO in each well was 0.05%v/v.

Ethane dimethane sulphonate (EDS) : EDS was prepared by Dr Rodney Kelly. EDS solutions were prepared using DMSO and M199E as described above. The final concentrations of EDS used were 2-230μM.

10.3. Results

KCZ : Concentrations of 0.1-100μg/ml KCZ had significant inhibitory effects (p <0.01) on basal testosterone production by rat Leydig cells (Figure 10.1.A.). Only the highest concentrations used (10-100μg/ml) significantly inhibited hCG-stimulated testosterone production by rat Leydig cells (p <0.01, Figure 10.1.B.). On 3 separate occasions concentrations of 0.1-100μg/ml KCZ significantly inhibited both basal and hCG-stimulated testosterone production by human Leydig cells (p <0.01, Figures 10.1.C. and D. and Table 10.1.).

HCZ : A concentration of 0.1 μg/ml HCZ had a significant stimulatory effect (p <0.05) on basal testosterone production by rat Leydig cells, while a concentration of 100μg/ml significantly inhibited basal testosterone production (p <0.01, Figure 10.2.A.). Variability in the response of isolated Leydig cells to hCG meant that no overall there was no effect of HCZ on hCG-stimulated testosterone production by rat Leydig cells (Figure 10.2.B.). On 3 separate occasions concentrations of 1-100μg/ml HCZ significantly inhibited both basal and hCG-stimulated testosterone

production by human Leydig cells (p <0.01, Figures 10.2.C. and D. and Table 10.2.).

TKD : Addition of 0.1-100μg/ml TKD had no effect on basal testosterone production by rat Leydig cells (Figure 10.3.A.). The highest concentration used (100μg/ml) significantly inhibited hCG-stimulated testosterone production (p <0.05, Figure 10.3.B.). On 3 out of 5 occasions 100μg/ml TKD significantly inhibited basal testosterone production by human Leydig cells, while on 2 out of 5 occasions, 1μg/ml TKD significantly stimulated basal testosterone production by human Leydig cells (Figure 10.3.C. Table 10.3.). A concentration of 100μg/ml TKD significantly inhibited hCG-stimulated testosterone production on 5 separate occasions. In 3 experiments 10μg/ml TKD also had a significant inhibitory effect (Figure 10.3.D. Table 10.3.).

EDS : Concentrations of 2 and 10μM EDS significantly stimulated basal testosterone production by both rat and human Leydig cells (Figures 10.4.A. and C.). Higher doses of EDS (up to 230μM) had no effect on testosterone production by rat Leydig cells, while 230μM EDS significantly inhibited basal testosterone production by human Leydig cells. None of the concentrations of EDS used had a significant effect on hCG-stimulated testosterone production by rat Leydig cells (Figure 10.4.B.). The effects of EDS on hCG-stimulated testosterone production by human Leydig cells, were comparable to the effects on basal testosterone production (Figure 10.4.D.).

Figure 10.1. The effects of KCZ on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=3). **B.** hCG-stimulated testosterone production by rat Leydig cells (n=3). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from control values.

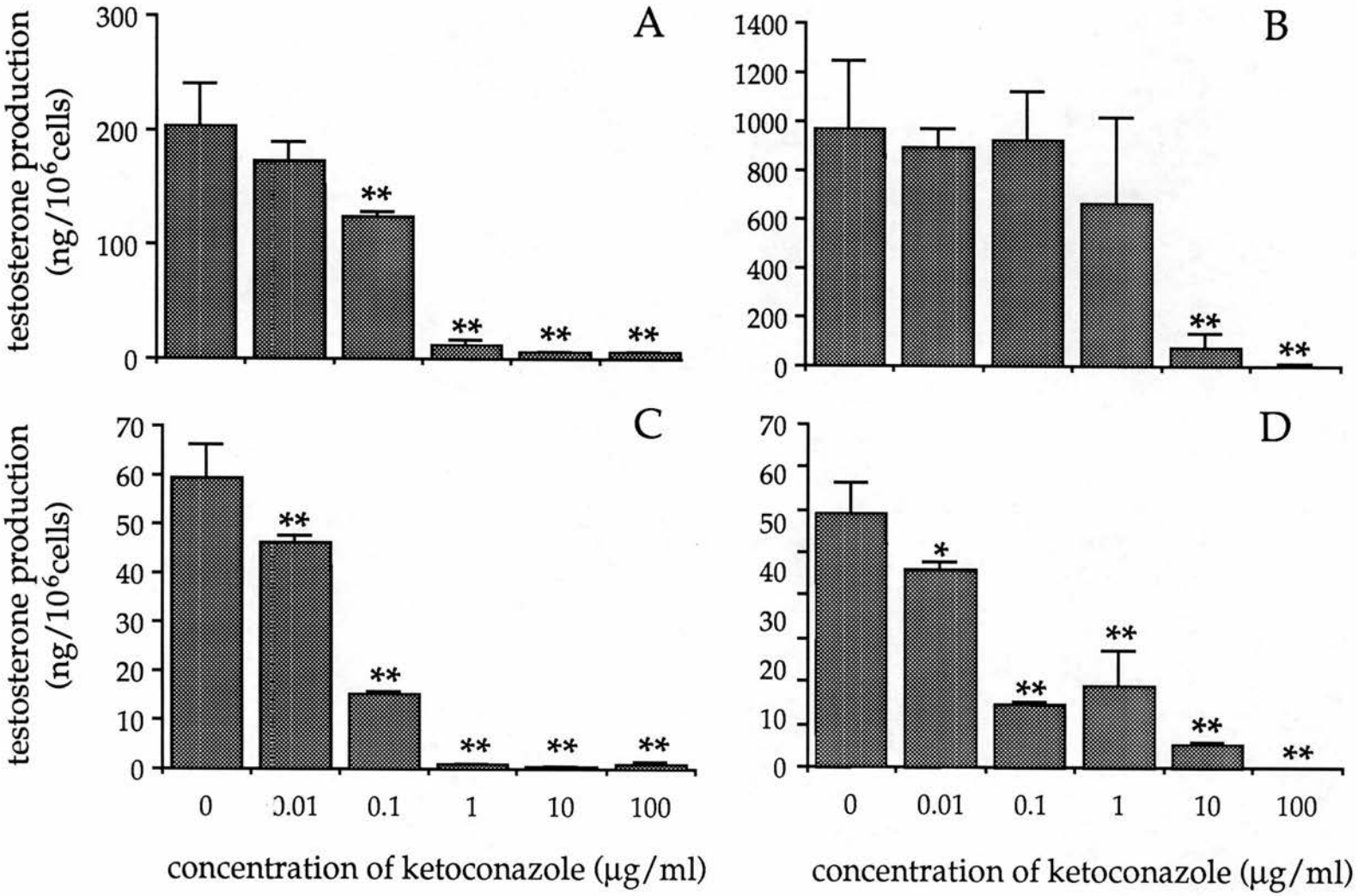

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
300
200
100
0
1400
1200
1000
800
600
400
200
0
70
60
50
40
30
20
10
0
**
*
0
0.01
0.1
1
10
100
concentration of ketoconazole (μg/ml)
concentration of ketoconazole (μg/ml)

Table 10.1. The effects of KCZ on testosterone production by human Leydig cells cultured for 20h.

The table shows the effects of KCZ on human Leydig cells in a further 2 experiments. The concentrations of KCZ are in μg/ml, and the amount of testosterone is given as ng/10^6 cells.

Each value is the mean±S.D. of triplicate. *p <0.05, **p <0.01, significantly different from control values, N.D. = not detectable.

A. Basal

control	0.01	0.1	1	10	100
61.1±2.3	119.9±54.13*	48.8	15.6±16.6	15.7±4.7	10.4±1.7*
37.14±6.2	37.3±1.9	20.5±3.0**	12.8±1.2**	N.D.	N.D.

B. hCG-stimulated

control	0.01	0.1	1	10	100
244.7±64.8	196.2±13.8	159.6±12.6**	34.8±5.2**	11.3±1.9**	11.5±0.8**
360.7±28.1	451.5±9.6*	258.3±9.6**	55.4±5.8**	N.D.	N.D.

Figure 10.2. The effects of HCZ on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=5). **B.** hCG-stimulated testosterone production by rat Leydig cells (n=5). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from control values.

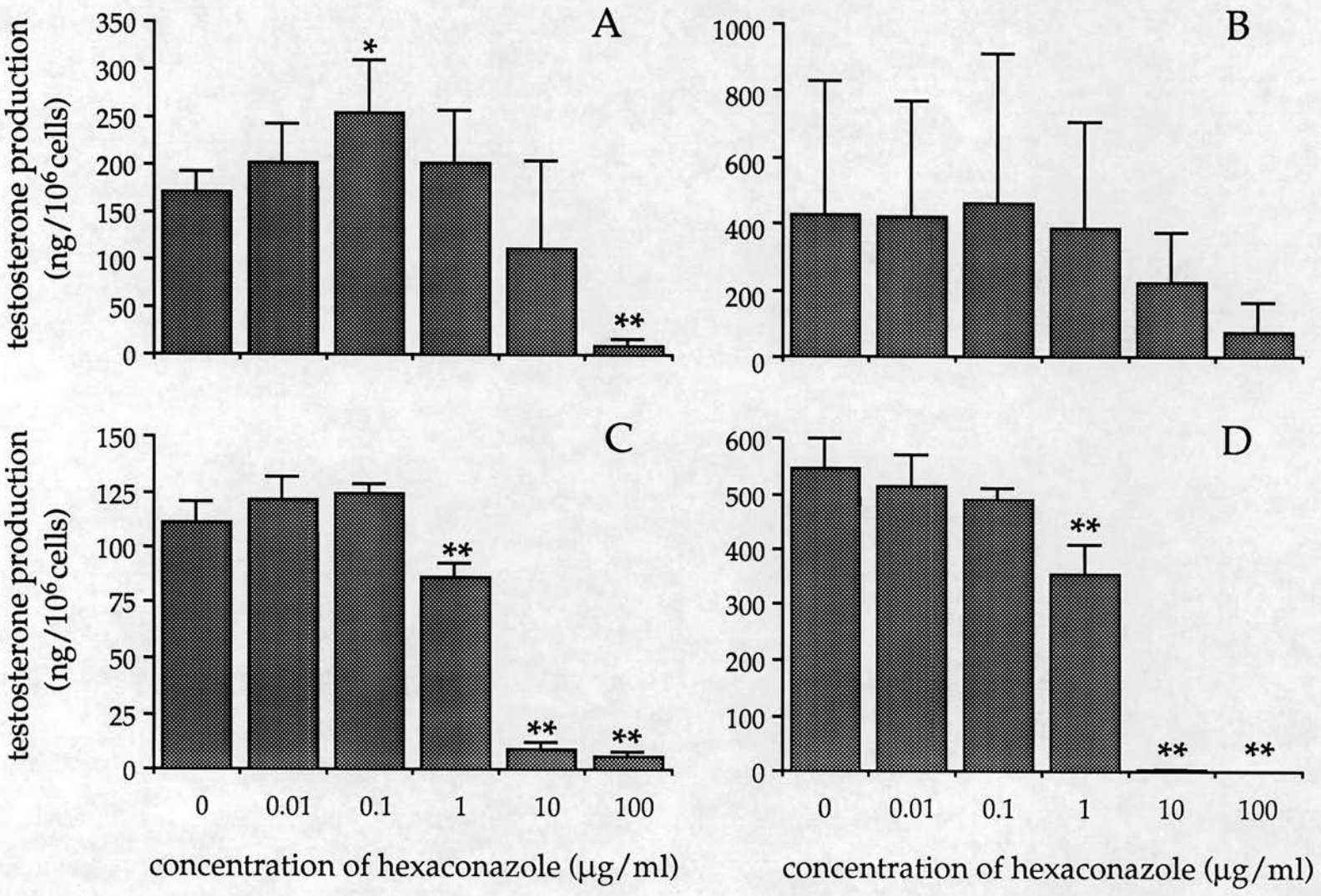

A
B
C
D
testosterone production (ng/10^6cells)
testosterone production (ng/10^6cells)
350
300
250
200
150
100
50
0
1000
800
600
400
200
0
150
125
100
75
50
25
0
600
500
400
300
200
100
0
*
**
**
**
**
**
**
0
0.01
0.1
1
10
100
concentration of hexaconazole (μg/ml)
concentration of hexaconazole (μg/ml)

Table 10.2. The effects of HCZ on testosterone production by human Leydig cells cultured for 20h.

The table shows the effects of HCZ on human Leydig cells in a further 4 experiments. The concentrations of HCZ are in μg/ml, and the amount of testosterone is given as ng/10^6 cells.

Each value is the mean±S.D. of triplicate. *p <0.05, **p <0.01, significantly different from control values, N.D. = not detectable.

A. Basal

control	0.01	0.1	1	10	100
61.1±2.3	111±84.9**	86.2±65.9	57.1±41.4	88.8±18.5	8.7±0.6**
32.6±1.2	35.6±2.5	33.7±3.4	16.03±2.5**	2.19±0.3**	N.D.
11.3±2.2	10.4±2.8	14.3±1.7	12.4±1.1	9.3±2	2.2±0.2**
43±10.1	32.1±3.7	35.9±2.9	25.9±0.5**	4.3±0.5**	N.D.

B. hCG-stimulated

control	0.01	0.1	1	10	100
261±69.1	216.5±27.8	195.8±5.12*	121±12.6**	70.3±9.1**	18.2±5.9**
165.7±4	_	124.5±44.5*	84.2±7**	10.6±1.5**	N.D.
42.5±5.7	45.6±9.2	52.6±8.5	53.9±7.6	43.5±7.1	25.3±4.4**
324.1±29.6	302.2±35.6	339.2±21.3	335.3±39.9	61±12.4**	0.4±.0.03**

Figure 10.3. The effects of TKD on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=5). **B.** hCG-stimulated testosterone production by rat Leydig cells (n=5). **C.** basal testosterone production by human Leydig cells (representative results). **D.** hCG-stimulated testosterone production by human Leydig cells (representative results). Each point is the mean±S.D. of n experiments with rat Leydig cells, and of triplicate wells for human Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from control values.

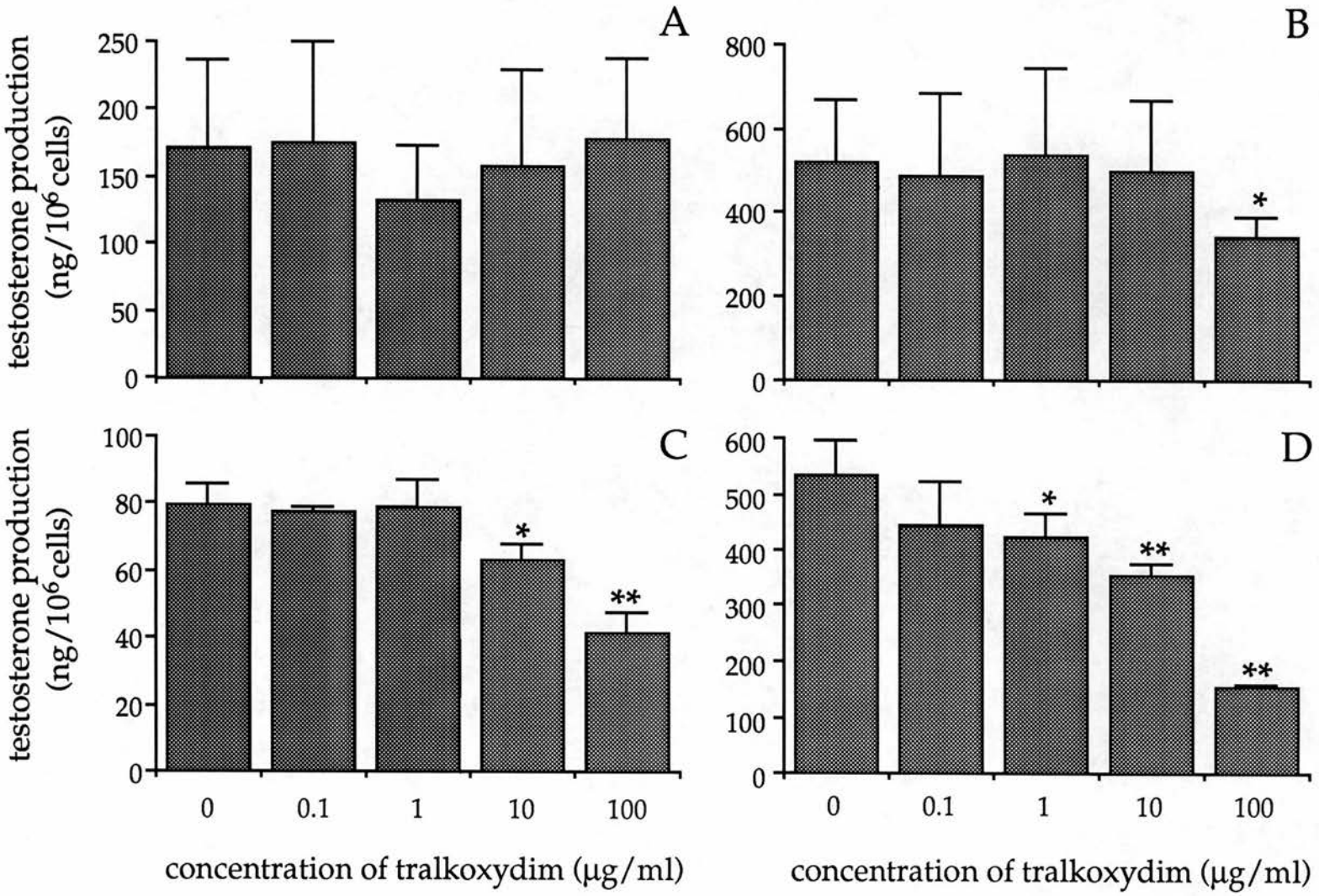

A
B
C
D
testosterone production (ng/10^6 cells)
testosterone production (ng/10^6 cells)
250
200
150
100
50
0
800
600
400
200
0
100
80
60
40
20
0
600
500
400
300
200
100
0
*
**
0
0.1
1
10
100
concentration of tralkoxydim (µg/ml)
concentration of tralkoxydim (µg/ml)

Table 10.3. The effects of TKD on testosterone production by human Leydig cells cultured for 20h.

The table shows the effects of TKD on human Leydig cells in a further 4 experiments. The concentrations of TKD are in μg/ml, and the amount of testosterone is given as ng/10^6 cells.

Each value is the mean±S.D. of triplicate. *p <0.05, **p <0.01, significantly different from control values.

A. Basal

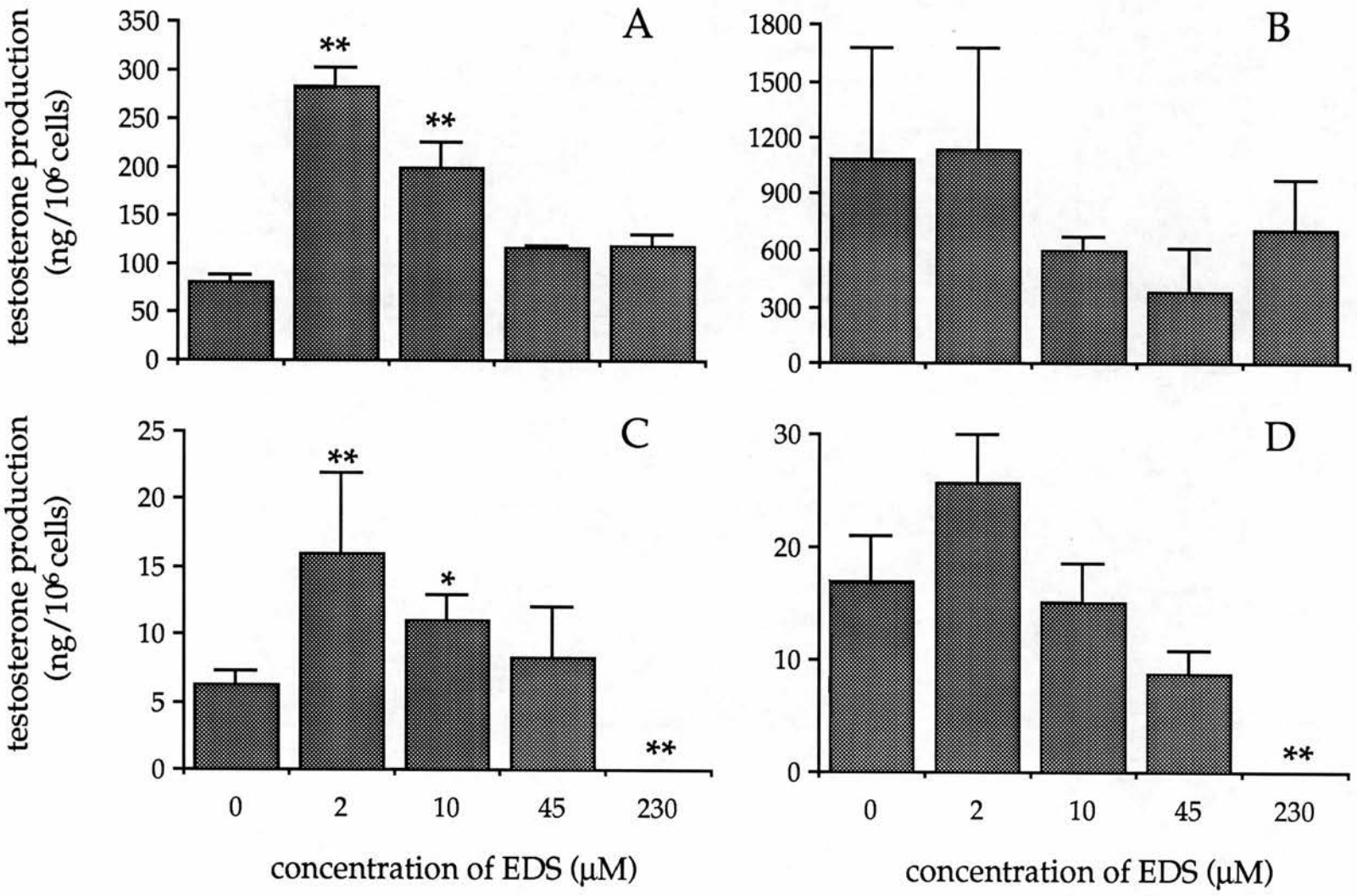
control	0.1	1	10	100
61.1±2.3	148.8±84.3*	180.1±23.2**	104.4±21.4	21.5±5.3
32.6±1.2	35.6±3.4	38±0.6**	35.1±2.8	20.1±0.5*
7.6±2.4	_	18.7±8.7	9±3.4	7.3±2.6
152.2±24.5	160.9±2.8	157.1±36.8	128.1±14.3	84.7±12.7**

B. hCG-stimulated

control	0.1	1	10	100
285.5±75.6	240±27.1	244.4±28.2	204.5±21.1*	170±12.5**
165.7±4	174.8±26.5	179±14	167.8±23	114.9±10.9**
16.9±4	13.4±3.1	14.3±5.4	22.4±3.3	6.9±2.6*
504.1±70.2	332.5±45.9*	327.1±47.4**	220±76**	217.1±.14.1**

Figure 10.4. The effects of EDS on testosterone production by rat and human Leydig cells cultured for 20h.

A. basal testosterone production by rat Leydig cells (n=3). **B.** hCG-stimulated testosterone production by rat Leydig cells (n=3). **C.** basal testosterone production by human Leydig cells (results of a single experiment). **D.** hCG-stimulated testosterone production by human Leydig cells (results of a single experiment). Each point is the mean±S.D. of 5 experiments with rat Leydig cells. $*p < 0.05$, $**p < 0.01$, significantly different from control values.


A
B
C
D
testosterone production (ng/10⁶ cells)
testosterone production (ng/10⁶ cells)
350
300
250
200
150
100
50
0
1800
1500
1200
900
600
300
0
25
20
15
10
5
0
30
20
10
0
**
**
**
*
**
**
0
2
10
45
230
0
2
10
45
230
concentration of EDS (μM)
concentration of EDS (μM)

10.4. Discussion

The experiments described in this chapter form a comparison of the toxic effects of 4 compounds on rat and human Leydig cell function.

KCZ is an orally active, imidazole derivative used clinically as an anti-fungal agent. KCZ has been shown to inhibit testosterone production both *in vivo* (Pont *et al.*, 1982) and *in vitro* (Albertson *et al.*, 1988, De Coster *et al.*, 1989). However, previous studies of the effects of KCZ on steroidogenesis in the human testis have used either crude interstitial cell preparations (De Coster *et al.*, 1989) or preparations of testicular microsomes (Albertson *et al.*, 1988). This is the first study to examine the effects of KCZ on purified human Leydig cells. In agreement with previous reports using rat Leydig cell preparations (Kan *et al.*, 1985, Sikka *et al.*, 1985), KCZ was found to inhibit both basal and hCG-stimulated testosterone production. In the rat inhibition is caused by blocking the action of cytochrome $P_{450}17\alpha$, which catalyses at least 2 successive reactions, namely 17α-hydroxylation and the 17-20 lyase reaction (Sikka *et al.*, 1985, De Coster *et al.*, 1989). Albertson *et al.* (1988) found that KCZ inhibited these activities in impure human Leydig cell microsomal preparations. However, De Coster *et al.* (1989) demonstrated preferential inhibition of 17α-hydroxylation in crude human testicular suspensions. The aims of the present study were purely comparative and examination of enzyme activities was not made. The study has confirmed that KCZ has an inhibitory effect on both basal and hCG-stimulated testosterone secretion by highly purified human Leydig cells. It has also been shown that human Leydig cells are more sensitive to the inhibitory action of KCZ, with significant inhibition first seen at 10-fold (basal testosterone) and 100-1000 fold (hCG-stimulated testosterone) lower concentrations in the human compared to the rat. De Coster *et al.* (1989) also found that testosterone biosynthesis was more sensitive to KCZ in the human than the rat.

Kan *et al.* (1985) used a rat model to test the steroidogenesis-inhibiting properties of several anti-fungal imidazoles, and demonstrated a cascade of potencies. To assess whether such differences were constant between species, this study also examined the effects of HCZ on

testosterone production by rat and human Leydig cells. HCZ is an anti-fungal triazole, which acts by inhibiting the action of cytochrome $P_{450}17\alpha$. HCZ had a significant inhibitory effect on basal testosterone production by rat Leydig cells while the inhibitory effect on hCG-stimulated testosterone production was not statistically significant. The effects of KCZ were seen at 0.1μg/ml, while no effect of HCZ was seen until concentrations of 100μg/ml were used. In the human HCZ inhibited both basal and hCG-stimulated testosterone production at concentrations of 1μg/ml and above. As with KCZ, human Leydig cells were more sensitive than rat Leydig cells to the effects of HCZ. In human Leydig cells, the first effects of HCZ were seen with a concentration 100 fold lower than that needed to produce similar effects in the rat. However, as with rat Leydig cells, HCZ was less potent than KCZ in inhibiting testosterone production in human Leydig cells. This suggests that when an animal model is required to represent steroidogenesis in the human, in order to assess the relative potencies of compounds which inhibit steroidogenesis, then the rat is a valid model.

To determine whether the validity of the rat model held when inhibition of testosterone production was not known to be via an effect on steroidogenic enzymes, a comparison was made between the effects of TKD and EDS on testosterone production in rat and human Leydig cells.

TKD is an antifungal agent which is unrelated to KCZ or HCZ. TKD had no effect on basal testosterone production by rat Leydig cells, although the highest dose used (100μg/ml) did inhibit hCG-stimulated testosterone production. In the human, basal testosterone production was inhibited by concentrations of 10μg/ml and above while hCG-stimulated testosterone production was inhibited by concentrations of 1 μg/ml and above. As with the imidazole compounds, human Leydig cells were 10-100 times more sensitive to the effects of TKD than were rat Leydig cells. The rat model was a reliable indicator of effects in the human, as in both species the effects of TKD were more pronounced on hCG-stimulated, rather than basal, testosterone production. The mechanism by which TKD exerts these effects is not known.

While the *in vivo* effects of EDS have been well documented (Kerr *et al.*, 1985, Jackson *et al.*, 1986, Morris *et al.*, 1986), nothing is known of its mechanism of action, and little of its direct *in vitro* effects. Rommerts *et*

al. (1988) demonstrated that 0.4mM EDS inhibited pregnenolone production by cultured rat Leydig cells. This study has shown that lower concentrations of EDS (2-10μM) can stimulate basal testosterone secretion by both rat and human Leydig cells. Concentrations of EDS up to 230μM had no effect on rat Leydig cells but caused complete inhibition of basal and hCG-stimulated testosterone production by human Leydig cells. Again human Leydig cells appeared to be more sensitive than rat Leydig cells to the adverse effects of EDS on steroidogenesis.

In summary, with all inhibitory compounds tested, rat Leydig cells proved to be an accurate, if less sensitive model for the actions of the compounds on human Leydig cell function.

11. General Discussion

Recently there has been increasing concern about the deleterious effects of environmental compounds on sperm counts in the male (Sharpe, 1992b, Carlsen *et al.*, 1992). Both studies emphasised the present lack of understanding of the mechanisms controlling spermatogenesis, especially in man. A further decline in sperm counts is unlikely to be prevented unless progress is made in elucidating testicular regulatory pathways. Spermatogenesis is driven by testosterone, thus the pathways that regulate testosterone production are potential sites for malfunctions that could result in impaired sperm output.

The studies presented in this thesis have examined the regulation of testosterone production in the human testis. Potential mechanisms of regulation of Leydig cell function *in vitro* and *ex vivo* were also investigated, using the rat as a model. Wherever possible comparisons were drawn between Leydig cell function in rat and man, in order to demonstrate when and how rat Leydig cells can be used as a model for man.

Simpson *et al.* (1987) described a technique for the isolation of human Leydig cells which were highly responsive to hCG. Previous studies using crude interstitial cell preparations had found human Leydig cells did not show a marked response to hCG *in vitro* (Huhtaniemi *et al.*, 1982). A frequent criticism concerning the use of human Leydig cells is that because they are obtained from an elderly population they may represent an abnormal population of cells in which the functional capacity has declined. The studies presented in chapters 4 and 5 oppose this idea. In a sample group which spanned four decades, the relative ability of human Leydig cells to produce testosterone *in vitro*, and to respond to hCG stimulation, did not decline as a function of increasing age. It was also established that the cells remaining at the end of the isolation procedure were representative of the cells found *in situ*. It was necessary to establish this, as two populations of Leydig cells are present in the human testis, distinguished by differential staining with toluidine blue into light and dark cells. Thus the techniques described in this thesis result in the isolation of human Leydig cell populations which are

equivalent to the populations found *in vivo*, which will produce testosterone *in vitro* and which can respond to hCG.

The existence of light and dark Leydig cells in the human testis is well documented (Schulze, 1984, Simpson *et al.*, 1987) but not understood. On the basis of studies presented in this thesis no distinction could be made between light and dark Leydig cells with respect to steroid production in vitro. Correlations made between the percentage of testicular volume occupied by various components revealed that light and dark Leydig cell cytoplasmic volumes did not correlate with the same factors. While the cytoplasmic volume of both Leydig cell types correlated with the volume of the testis occupied by germ cells and the volume of the interstitium, only the cytoplasmic volume of dark Leydig cells correlated with tubular lumen volume. Light Leydig cell cytoplasmic volume showed specific correlations to the volume of peritubular tissue and the volume of blood vessels in the interstitium. These distinct correlations may be the initial evidence for differential roles for light and dark Leydig cells in mediating intratesticular interactions.

Rat and human Sertoli cells *in vitro* secrete a factor(s) which can stimulate testosterone production by isolated Leydig cells (Verhoeven & Cailleau, 1985,1986, 1990; Papadopoulos, 1991). There is no definitive information on the nature of the communicators between Sertoli cells and Leydig cells, yet there are several factors which have been shown to affect the function of isolated rat Leydig cells. Two factors for which there is additional evidence for a Sertoli cell site of production are testicular LHRH and AVP (see Sharpe, 1992 for review). Human Leydig cells were shown to respond to AVP and an LHRH agonist in a very similar manner to rat Leydig cells, suggesting that if AVP and LHRH have a role in mediating the regulation of Leydig cell function by Sertoli cells, it is a mechanism that is conserved across species. However, the role of AVP and LHRH as paracrine regulators of testicular function is not an established fact. Previous studies *in vivo* have not supported a paracrine role for LHRH in the human testis, based on the inability of LHRH-A to alter hCG-stimulated testosterone level (Rajfer *et al.*, 1987). Also, the effects of AVP reported in chapter 6 were small.

However, experiments such as those described in chapter 6 may give only a restricted view of a complex picture. There are several

examples in the literature of how germ cells can influence Sertoli cell function. Recently Onoda & Djakiew (1991) have demonstrated that germ cells influence the secretion of a Sertoli cell factor(s) which can stimulate steroidogenesis in isolated Leydig cells. However, such an *in vitro* approach has many limitations, not least of which is whether it is really relevant to the complex *in vivo* situation. The results of these *in vitro* experiments, which mixed populations of cells isolated from mature and immature animals, have been confirmed in the present study. The influence of germ cells on Sertoli cell-Leydig cell communication was determined by using MAA to chemically deplete specific populations of germ cells *in vivo* and examining Leydig cell function *in vitro*. The advantages of this technique are that it avoids using Sertoli cells from immature rats, which must be used in *in vitro* experiments, and it leaves all other pathways of intercellular communication intact, limiting the disruptive effect on testicular function. The induced absence of pachytene spermatocytes or round spermatids *in vivo* was associated with increased testosterone production *in vitro* by Leydig cells which were isolated from these testes. In contrast, the *in vivo* absence of elongated spermatids was not associated with any change in the subsequent function *in vitro* of isolated Leydig cells. Thus, it appears that the absence of germ cells, specifically pachytene spermatocytes or round spermatids, is associated with the removal of an inhibitory influence on Leydig cell function. The alternative interpretation, that the depletion of either germ cell type leads to the secretion of a stimulatory factor, is not supported by the *in vitro* work of Onoda *et al.* (1991) who demonstrated that both pachytene spermatocyte or round spermatid conditioned medium could inhibit protein production by isolated Sertoli cells.

Surprisingly, germ cell influences on Leydig cell function appeared to be related to the absence/presence of pachytene spermatocytes or round spermatids from seminiferous tubules at certain stages of the spermatogenic cycle. In particular, the absence of pachytene spermatocytes or round spermatids at stage I appeared to be associated with increased testosterone production by Leydig cells isolated from affected testes. When Leydig cells were either co-cultured with segments of seminiferous tubules at specific stages of the spermatogenic cycle, or when conditioned medium prepared from staged seminiferous tubules was added to isolated

Leydig cells the presence of stage II-V tubules inhibited basal testosterone production. The results of these experiments indicate that the presence of germ cells from *early* stages of the spermatogenic cycle may lead to inhibition of Leydig cell function. Together these experiments provide new evidence for an inhibitory influence of the early spermatogenic stages on testosterone production.

Stage VII is the most androgen-dependent stage (Russell & Clermont, 1977, Bergh, 1983, Sharpe *et al.*, 1992), and the study examining the extent to which cultures of seminiferous tubules metabolised exogenous testosterone demonstrated that the degree of metabolism was greatest at stages VI-VIII, a finding perhaps reflecting the greater utilisation of testosterone by androgen-dependent stages.

The metabolism of known amounts of exogenous testosterone added to cultures of staged seminiferous tubules was unaffected by the absence of a normal complement of pachytene spermatocytes in the tubules (chapter 9). Therefore, if pachytene spermatocytes do have a negative influence on the levels of testosterone measured in Leydig cell/seminiferous tubule co-cultures, it is not via a stimulatory effect on the rate at which seminiferous tubules metabolise testosterone.

The experiments presented in this thesis examining the regulation of steroidogenesis in the rat testis, have all supported the idea that local control mechanisms are specific to particular stages of the spermatogenic cycle. If this specificity is taken into consideration it could explain the difficulty in demonstrating the relevance *in vivo* for an action described *in vitro*. For example, administration of LHRH antagonists has little effect on intratesticular testosterone concentrations (see Sharpe, 1986 for review). The results described in this thesis suggest that steroidogenesis is regulated by local events occurring around each segment of a tubule at a specific stage. If there are 17.4m of tubules in each rat testis (Wing & Christensen, 1982), then the result of a paracrine effect on seminiferous tubules at one or two particular stages would probably not be reflected by any change in total testicular levels of testosterone due to 'dilution' of any effect. An attempt to establish the extent of regulation of Leydig cell function, could be made by isolating Leydig cells from rats in which the seminiferous tubules had been stage-synchronised by induction of vitamin A deficiency (Griswold *et al.*, 1989). The effects of potential

paracrine agents such as AVP and LHRH could then be examined on Leydig cells which *in vivo* had only been exposed to the influence of one spermatogenic stage. The use of MAA to deplete specific germ cells *in vivo* (chapter 7) has already shown that the influence of the *in vivo* environment is reflected in the performance of Leydig cells when isolated. Leydig cells isolated from stage-synchronised testes could be co-cultured with seminiferous tubules prepared from rats lacking selective germ cell types. This could make the elucidation of testicular intercellular communication pathways more straightforward.

If it is true that the germ cell complement at a given stage determines Sertoli cell function and thus potentially has a secondary effect on Leydig cell function (Sharpe, 1986), then, differences in local control mechanisms in rat and man might be expected. In the rat stages are arranged into waves which progress sequentially along the length of a tubule. In the human the spermatogenic wave proceeds along a spiral, so that each cross-section of a tubule contains germ cells from several different stages (Schulze & Rehder, 1984). These differences in spatial organisation may either be a consequence or a cause of different local regulatory mechanisms. In this context it is interesting to recall the presence of light and dark Leydig cells in the human testis, described in chapters 4 and 5. No correlation was found between the relative proportions of light and dark cells and the ability of Leydig cells to produce testosterone *in vitro*. However, the percentage volume of light and dark Leydig cells did not correlate with the same testicular cell types or structures. As evidence from the rat increasingly supports stage-specific synchrony of events in both the seminiferous epithelium and the interstitium (Bergh, 1983, McKinnell & Sharpe, 1992 and chapters 7 and 9) it would be of great interest if light and dark Leydig cells were found to be associated with specific and different stages of the spermatogenic cycle. This would require painstaking histological analysis of serial-sectioned testicular tissue.

An approach that could be used to determine stage specific effects is *in situ* hybridisation. A study that would be relevant to some of the results presented in this thesis would involve mRNA probes to the newly sequenced GnRH receptor (Tsutsumi, 1992). By using probes to localise both ligands and their receptors, the production or not of

testicular GnRH by Sertoli cells, and the presence or absence of GnRH receptors on Leydig cells could be finally determined if expression of the mRNA occurs in sufficient amounts. In addition, *in situ* hybridisation would allow stage-specific localisation of GnRH mRNA, implying stage-specific production and action. This technique could be applied to Leydig cell and Sertoli cell products and would allow comparisons between rat and man to be made relatively easily. Furthermore, as it does not appear to be possible to physically separate light and dark Leydig cells, *in situ* hybridisation and immunocytochemistry are possibly the only techniques which could be used to discover whether light and dark Leydig cells do have different functions.

Although the arguments presented so far have supported the existence of very specific, local interactions between Leydig cells and Sertoli cells, and on this basis could imply that the rat Leydig cell may not be a good model for the human Leydig cell, there is plenty of evidence to the contrary. The experiments presented in chapters 6 and 10, examining the effects of various compounds on Leydig cell function have demonstrated that *in vitro*, rat and human Leydig cells function in a very similar manner. AVP and LHRH-A stimulated basal testosterone production in both rat and human Leydig cells, while ANP did not affect either type of Leydig cell. The one difference found between the two species was that rat Leydig cells responded to the presence of diazepam or DBI, while human Leydig cells did not. However, the actions of DBI are probably concerned with intracellular regulation of steroidogenesis (see chapter 6), so are perhaps not relevant to discussions of intercellular control mechanisms. The effects of various toxicants on testosterone production by rat and human Leydig cells were remarkably similar, though human Leydig cells were generally more sensitive than rat Leydig cells.

The high degree of comparability between the patterns of radio-labelled proteins secreted by cultured rat and human Leydig cells further emphasises the similarity between the way rat and human Leydig cells function *in vitro*. These similarities would be presumed to extend to *in vivo* function. Results such as these indicate that the rat Leydig cell is an excellent model for studying human Leydig cell function *in vitro*.

The intentions of the studies reported in this thesis were to examine potential mechanisms of regulation of human Leydig cell function *in vitro* and at the same time to compare the regulation of rat and human Leydig cells *in vitro*. The study of intratesticular control in the rat emphasised the stage-specific nature of the testicular control mechanisms. When direct comparisons were made between rat and human Leydig cell function *in vitro*, Leydig cells from the two species were found to be remarkably similar, suggesting the similarity may extend to local control mechanisms. This is indirectly supported by the morphometric analysis of human testicular sections. As no simple relationship was found between the numbers of light or dark Leydig cells per testis and testosterone production in vitro, a more complex relationship including other cell types might be implied. The role of germ cells in altering Leydig cell production, shown experimentally in the rat, could be determined in man by relating morphometric data on germ cells to subsequent Leydig cell testosterone production *in vitro*. To do this, testicular sections need to be re-examined and different germ cell types quantified so that the possible relationship between the absence/presence of specific germ cell types and Leydig cells can be assessed.

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Patient no.	Age	Weight (g)	cell yield	basal testosterone	hCG-stim testosterone	fold response to hCG
1	54	31.99	7.43	65.7	270	4.1
2	68	25.82	2.18	32.6	165.6	2.9
3	69	20.4	2.18	3.01	8.85	2.9
4	64	26.51	3	4.13	13.57	3.3
5	89	23.43	2.01	11.9	28.66	2.4
6	80	21.61	4.35	7.57	16.86	2.2
7	63	30.14	6.83	79.73	535.9	6.7
8	73	37.02	2.18	87.56	253.68	2.9
9	70	23.8	14.7	48.19	639.92	13.3
10	68	14.95	4.3	11.25	42.52	3.8
11	65	11.76	2	26.75	114.38	4.3
12	56	26.89	13.7	163.62	870.41	5.3
13	82	23.35	9.8	108.02	497.45	4.6
14	74	18.59	6.45	59.48	314.98	5.3
15	68	24.03	5.4	37.14	360.66	9.7
16	86	17.8	5.05	120.35	365.8	3
17	79	28.21	10.5	273.35	1169	4.3
18	70	52.81	5.85	207.1	308	1.5
19	88	20.62	6.08	7.3	33.6	4.6
20	76	21.88	3.96	72.6	361.5	5
21	75	6.6	3.15	210	1630	22.5
22	57	59.34	10.98	124.2	1012.6	8.2
23	78	37.25	4.98	0.27	5.6	20.7
24	66	12.07	3.2	0.5	1.8	3.6
25	73	27.55	5.5	9.4	16.6	1.8
26	85	30.29	5.76	46.82	384	8.2
27	70	43.46	4.1	52.8	76.8	1.5